TESTING AND EVALUATION OF BIOACTIVE COMPOUNDS IN SOYBEAN

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ABSTRACT

This study was aimed to estimate the antioxidants, antibacterial, and antifungal activities of methanolic extract from soybean seeds against pathogenic microorganisms. The methanolic extract was prepared from defatted soybean flour. Total phenolics content (TPC), total flavonoid content (TFC), and the major phenolic, flavonoid, and isoflavone compounds in soybean extract were estimated by HPLC. On the other hand, biological activities such as antioxidants, antibacterial, and antifungal activities were evaluated. The value of phenolic compounds was 8.59 mg GAE/g extract, while the value of flavonoids was 0.82 mg QE/g extract. The chemicals listed below were chromatographed and identified: syringic acid, quercetin, gallic acid, benzoic acid, genistein, daidzein, p-coumaric acid, glycitein, and ferulic acid. The methanolic extract showed the antibacterial and antifungal activity against tested microorganisms. Considering the results, it is possible to employ the methanolic extract from soybean seeds, which is rich in phenolic chemicals, as an antioxidant, antibacterial, and antifungal agent. It functions well as a pure, natural product.

Keywords: soybean flour; phenolic compounds; methanolic extract; antifungal; antioxidant

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إختبار وتقييم المركبات النشّطة بيولوجياً في فول الصويا

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المستخلص

لتقدير الأنشطة المضادة للأكسدة والمضادة للبكتيريا والفطريات لمستخلص الميثانول من بذور فول الصويا ضد الكائنات الحية الدقيقة المسببة للأمراض. تم تحضير المستخلص الميثانولي من دقيق فول الصويا منزوع الدهن. تم تقدير إجمالي محتوى الفينولات (TPC)، إجمالي محتوى الفلافونويد (TFC)، ومركبات الفينول والفلافونويد والأيسوفلافون الرئيسية في مستخلص فول الصويا بواسطة HPLC. من ناحية أخرى، تم تقييم الأنشطة البيولوجية مثل مضادات الأكسدة والأنشطة المضادة للبكتيريا والفطريات. كانت قيمة المركبات الفينولية 8.59 ملجم من مستخلص GAE/ جم، بينما كانت قيمة الفلافونويد 0.82 ملجم من مستخلص QE/ جم. تم تحديد المواد الكيميائية المدرجة أدناه كروماتوجرافيك وتحديدها: حمض السرينجيك، كيرسيتين، حمض الغاليك، حمض البنزويك، الجينيستين، الديدزين، حمض الكوماريك، الجلايسيتين، وحمض الفيروليك. أظهر المستخلص الميثانولي النشاط المضاد للبكتيريا والفطريات ضد الكائنات الحية الدقيقة المختبرة. بالنظر إلى النتائج، من الممكن استعمال المستخلص الميثانولي من بذور فول الصويا، الغني بالمواد الكيميائية الفينولية، كمضاد للأكسدة، ومضاد للبكتيريا، ومضاد للفطريات. إنه يعمل بشكل جيد كمنتج طبيعي نقي.

الكلمات المفتاحية: فول الصويا، مركبات الفينول، مستخلص الميثانول، مضاد للفطريات، مضاد للأكسدة

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INTRODUCTION

Many edible plants, including many oil-producing plants like soybean (*Glycine max*), canola, flaxseed, and olive that are utilised as food or sources of food ingredients, include phenolic compounds, which are now recognised as crucial minor components of many of these plants. Oil-bearing plants have historically been employed as significant sources of both protein and oil. Some examples include soybean, canola, flaxseed, and olive (6, 15). It has been documented how phenolic chemicals function as antioxidants to protect bodily tissues from oxidative damage, preserve the nutritional value of food and food products, and retain the quality of food (7). Reactive oxygen species (ROS) and other free radicals with an oxygen core are produced by several physiological processes in the human body. ROS contributes to the manufacture of molecules with biological significance, phagocytosis, the control of cell development, and intercellular signaling. However, excessive ROS generation can also be damaging to the body since it can cause DNA mutation, membrane protein degradation, and cell membrane disintegration, all of which can contribute to the onset or progression of numerous disorders (13, 27). Antioxidants are substances that, by scavenging free radicals and reducing oxidative stress, can delay, inhibit, or prevent the oxidation of oxidizable materials. Numerous antioxidant phytochemicals or bioactive compounds found in plants can neutralize free radicals and so slow the progression of many chronic diseases linked to oxidative stress and ROS. Consuming natural antioxidants has been linked to a lower risk of diabetes, cancer, cardiovascular disease, and aging-related disorders. Phenolic compounds and other naturally occurring phytochemicals have the potential to act as antioxidants, according to studies on dietary molecules that scavenge free radicals (8). Soybean isoflavones include antimicrobial and antioxidant action, and their antibacterial activity has received much research. Numerous studies have shown that isoflavones derived from soybeans can stop the growth of numerous microorganisms (19). Over the past decades, interest in pharmacology and herbal medicine has expanded due to the usage of alternative medicine therapies. Novel medicinal molecules have been inspired by plants since plant-derived medications have greatly improved human health and wellbeing. The marketable agricultural plant known as soybean (*Glycine max*) is used to make a variety of goods including edible soybean oil, soy milk, tofu, soy flour, roasted soybeans, and numerous processed goods. Not only is soybean processed for use in the human food industry, but it is also extensively employed in the manufacture of animal feed, cosmetics, and biodiesel. The content of soybeans was analysed, and the results revealed significant amounts of fatty acids, a wide range of vitamins, and minerals in addition to a great variety of amino acids that are crucial for human health. Soybean has a sizable group of bioactive phytochemicals, a class of chemical substances originating from plants. The three main groups are coumestans, lignans, and isoflavones. Soybean contains a high amount of isoflavones from these three classes (16). In the current study, the methanolic extract was prepared from defatted soybean flour. Total phenolics content (TPC), total flavonoid content (TFC), and the major phenolic, flavonoid, and isoflavone compounds in soybean extract were estimated by HPLC. On the other hand, biological activities such as antioxidants, antibacterial, and antifungal activities were evaluated.

MATERIALS AND METHODS

Methanolic extract preparation

Soybean seeds were bought from the neighborhood market. Using a Soxhlet device and hexane, soybean seeds were crushed and defatted for eight hours. Phenolic compounds were extracted from the raw, defatted seeds using a Soxhlet apparatus and 70% (v/v) aqueous methanol at a solid-to-solvent ratio of 1:5 for 2 h. The extracts were separated by centrifugation and concentrated in a rotary evaporator below 40 °C. The resulting aqueous solutions were lyophilized and stored at -20 °C until used.

Total phenolic content determination

The Folin-Ciocalteu method was employed to determine the amount of total phenolics (10). 0.5 mL of extract (1000 ppm) was mixed with 5 mL of distilled water, and 0.5 mL of the

Folin-Ciocalteu reagent. 1.0 mL of saturated sodium carbonate solution was added after 3 minutes had passed. After shaking, this mixture was let to stand for a one hour. Using a Hitachi UV-Vis U 3000 spectrophotometer (Tokyo, Japan), the absorbance was measured at 725 nm (each measurement was done three times). The results were reported as mg GAE (gallic acid equivalents)/g dry extract using a calibration curve for gallic acid.

Total flavonoid content determination

According to Lamaison and Carnat, the total flavonoid content was measured spectrophotometrically (22). Briefly, 0.5 mL extract (1000 ppm) was combined with 0.5 mL of 2% aluminum chloride dissolved in ethanol. After one hour, 415 nm absorption measurements were made in comparison to a blank (ethanol). Using a standard curve with quercetin, the total flavonoid content was calculated. The amount of quercetin equivalents (QE/g of dry extract) was calculated using the average of three readings.

HPLC analysis of phenolic compounds

Soybean seeds were extracted with methanol, and the polyphenolic compounds were identified using High-Performance Liquid Chromatography (HPLC). A C18 column (125 mm 4.60 mm, 5 m particle size), two LC pumps, and a UV/Vis detector make up the HPLC-Agilent 1100 equipment (2).

Antioxidant activity (DPPH-assay)

Antioxidant activity of soybean seeds methanolic extract was estimated through the use DPPH-assay according to (24). Briefly, 2 mL of a freshly made methanolic solution of DPPH (1 mM, 0.25 mL) was combined with 0.1 mL of a soybean methanolic extract at various concentrations (100, 200, 400, and 800 µg extract/ml). After being vortexed, the mixture was let to sit at room temperature for 30 minutes. The absorbance was measured with a spectrophotometer at 517 nm. The antioxidant activity was calculated from the following equation:

$$\boldsymbol{Antioxidant\ activity}\ (\%) = [(\boldsymbol{Abs.control - Abs.sample})/\ \boldsymbol{Abs.control}]\ \boldsymbol{x\ 100}$$

The data were expressed as half the maximum effective concentrations to quench the DPPH (EC_{50}).

Antibacterial activity

Gram-positive (*Listeria monocytogenes* and *Bacillus thuringiensis*) and Gram-negative bacteria (*Escherichia coli* and *Pseudomonas fluorescens*) were used to examine the methanolic extract of soybean seeds for their antibacterial properties. By using a disc diffusion experiment, antibacterial activity was assessed, according to (21). A loop of single colonies on Muller Hinton agar (MHA) plates was suspended in 10 mL Muller Hinton broth (MHB), incubated at 37 °C, and the absorbance at 600 nm was measured to obtain 0.5 McFarland turbidity ($1.5x10^8$ CFU/mL). Saturated discs (6 mm) with methanolic extract concentrations (5, 10, 20, 50, and 100 µg/mL) were placed in Muller Hinton agar (MHA) Petri dishes previously inoculated with harmful microorganisms. The MHA Petri dishes were incubated for 24 hours at 37 °C. The Ruler was used to calculate the size of the inhibition zones surrounding the discs (mm).

Antifungal activity: The impact of soybean seeds methanolic extract at different concentrations (5, 10, 20, 50, and 100 µg/mL) on Pythium spp. growth on a potato dextrose agar (PDA) medium was examined as described by (1, 9). At 25 °C, plates were incubated in the incubator. Daily measurements of colony diameters continued until the control Petri plates were entirely covered by the fungus. The equation below can be used to calculate linear growth.

$$\mathbf{LGR}\ (\%) = [(\mathbf{CG - TG})/(\mathbf{CG})]\ \mathbf{x\ 100}$$

LGR: linear growth reduction; CG: control growth; TG: treatment growth

Scanning electron microscopy (SEM)

Pathogenic fungi were handled for 4 hours at 25 °C with methanolic extract at a concentration of 100 µg/mL compared to the control according to (26).

RESULTS AND DISCUSSION

Total phenolic contents and total flavonoids contents in soybean seeds methanolic extract : In the present study, soybean seeds methanolic extract was prepared and analyzed for total phenolic content (TPC), and total flavonoid (TF), and the results show in Figure 1. The value of phenolic compounds was 8.59 mg GAE/g extract, while the value of flavonoids was 0.82 mg QE/g extract. According to (Abd Elhamid, et al., 2022) there was considerable heterogeneity for the TPC

and TF, and the cultivars varied greatly in terms of these variables. According to (Abd Elhamid, et al., 2022), TPC levels in seed extracts range from 6.4 mg GAE/g extract in Giza 22 to 10.5 mg GAE/g extract in Giza 35. TFC ranged between 0.55 mg QE/g extract in Crawford and 1.20 mg QE/g extract in Giza 111. TPC concentrations in seed extracts range from 10.3 to 13.7 mg/g. Cv. Mazowia has the greatest concentration of phenolic compounds among the examined cultivars (13.07 mg/g of extract). The cultivars Satina (12.5 mg/g of extract), Augusta (12.4 mg/g of extract), and Progres (12.04 mg/g of extract) had lower phenolic contents. Even less phenolics (11.7 mg/g extract) were present in the Polish cv. Aldana seed extract. The French variety Isidor has the lowest TPC of all the cultivars analysed (10.3 mg/g of extract) (18).

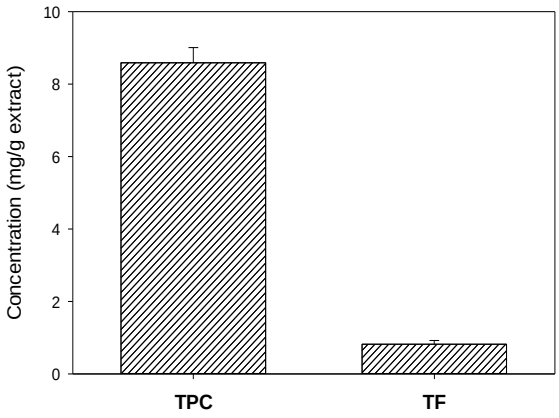

Figure 1. Total phenolic (TPC) and total flavonoid contents (TF) in soybean methanolic extract. All data are expressed as the mean (n = 3) with ±SD

Identification of phenolic, flavonoid, and isoflavone compounds by HPLC

HPLC was used to separate the phenolic components in the soybean seed extract. The results are in Figure 2 indicate the chemicals listed below were chromatographed and identified: syringic acid, quercetin, gallic acid, benzoic acid, genistein, daidzein, p-coumaric acid, glycitein, and ferulic acid. The concentrations of identified compounds show in Table 1. Quercetin was recorded the highest value (29.6 %) followed by genistein (27.8%), and daidzein (13.6%). An HPLC chromatogram shows the isoflavone compounds (genistein, daidzein, and glycitein). Previous research has found that soybean contains a variety of phenolic acids, including syringic, ferulic, sinapic, p-coumaric, hydroxybenzoic, caffeic, and chlorogenic acids, and that these total phenolic compounds are highly positively correlated with hydroxybenzoic acids, including gentisic acid and salicylic acid (2, 12).

Table 1. Major phenolic, flavonoid, and isoflavone constituents (%) as determined by HPLC

Compounds	Concentration (%)
Syringic acid	**0.91**
Quercetin	**29.6**
Gallic acid	**6.76**
Benzoic acid	**3.47**
Genistein	**27.8**
Daidzein	**13.6**
***p*-Coumaric acid**	**3.2**
Glycitein	**5.9**
Ferulic acid	**4.2**

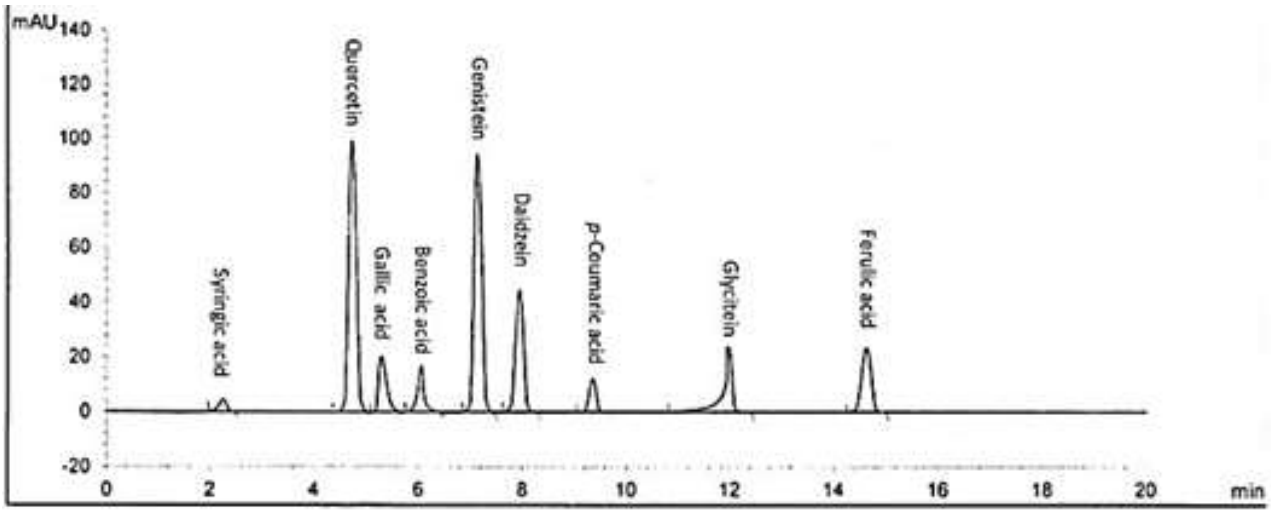

Figure 2. Major phenolic, flavonoid, and isoflavone constituents as determined by HPLC

Antioxidant activity

In vitro test using the DPPH radical was used to investigate the ability of produced soybean seed extract to scavenge free radicals. The results are presented in **Figure 3**.

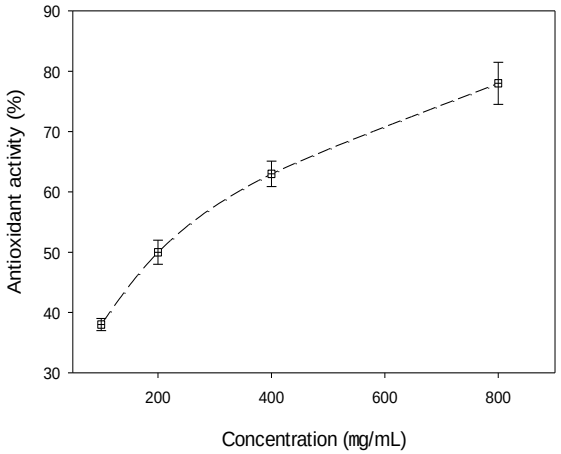

Figure 3. Antioxidant activity (%) of soybean seeds methanolic extract at different concentrations (100, 200, 400, and 800 µg/mL) using DPPH-assay

The EC_{50} value was observed at 200 µg/m. Concentration-dependent increases in antioxidant activity are showed. The antioxidant activity of soybean seeds is due to their increased content of phenolic compounds. It has been discovered that isoflavones, a kind of flavonoid present in soybean seeds, contain significant secondary chemicals with a variety of chemical activities. They appear to have particularly beneficial antioxidant properties (17). Numerous studies have shown a link between isoflavone concentration and the antioxidant activity of soybeans (4, 20). Approximately 72% of all phenolic chemicals in soybean seeds are isoflavonoids (25). Isoflavones are exceptionally potent antioxidants, especially when they are present in their natural state. Genistein and dadzein, two isoflavones found in foods, have the highest antioxidant potency (11).

Antibacterial activity

Table 2 and Figure 4 shows the methanolic extract from soybean seeds antibacterial activity against *L. monocytogenes*, *Staph. aureus*, *E. coli*, and *P. flurocences* at various concentrations. Extract at 5 µg/mL produced no inhibition zones against all tested bacteria. Extract at 10, and 20 µg/mL produced no inhibition zones against *P. flurocences.* These activities were mainly associated with some bioactive secondary compounds, such as polyphenols, alkaloids, and phytosterols, as well as some nutritional primary compounds, such as proteins and glycosides (3).

Table 2. Antibacterial activity of soybean seeds methanolic extract at different concentrations (5, 10, 20, 50, and 100 µg/mL)

Concentration (µg/mL)	Inhibition zone diameter (mm)			
	L. monocytogenes	*Staph. aureus*	*E. coli*	*P. flurocences*
5	0	0	0	0
10	6	8	5	0
20	13	21	13	0
50	18	26	22	12
100	28	35	36	16

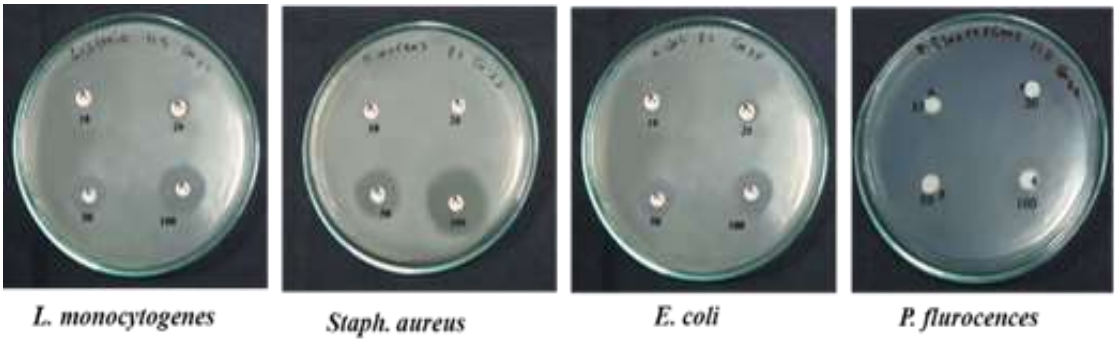

Figure 4. Antibacterial activity of soybean seeds methanolic extract at different concentrations against tested bacteria

Antifungal activity

Linear growth (Cm) and growth reduction (%) of *Pythium* spp. grown on solid agar medium after 7 days at 25 °C in the presence of soybean seeds methanolic extract at several concentrations are show in Figure 5.

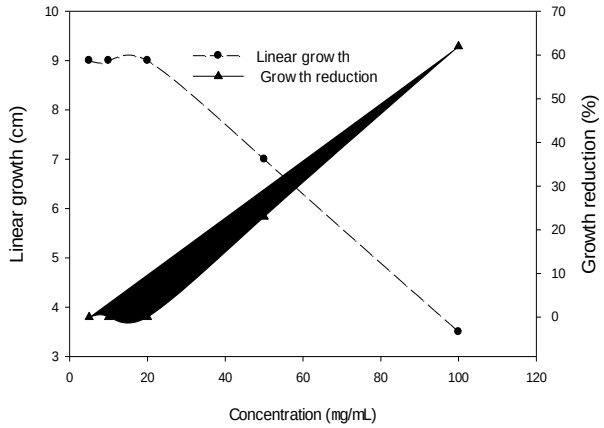

Figure 5. Linear growth (Cm) and growth reduction (%) of *Pythium* spp. grown on solid agar medium after 7 days at 25 °C in the presence of soybean seeds methanolic extract at several concentrations

The presence of soybean seeds' methanolic extract evidently reduced the mycelial growth of Pythium spp. in a concentration-dependent manner. The fungal growth of Pythium spp. was reduced by 23.3% and 62% in response to soybean seeds' methanolic extract application at 50, and 100 µg/mL, respectively. Pythium spp SEM pictures after being subjected to soybean seeds methnolic extract (100 µg/mL) for 4 hours at room temperature are shows in Figure 6. The untreated, unmodified fungus revealed typical hyphae with walls that seemed to be unbroken. The SEM picture of the typical Pythium Spp untreated fungal conidia appeared to be quite uniform and pear-shaped. The structural characteristics of both fungal hyphae and conidia have been dramatically altered by treatment with soybean seeds methnolic extract, entirely destabilising and deforming this shape at (100 µg/mL). The two processes outlined above can be inhibited by phenolic compounds, which are produced as secondary metabolites and whose profile and quantities are proportionate to the stress experienced by plants during their life cycles (23). The vacuoles of plant cells contain soluble free phenolic compounds, whereas soluble conjugated forms are bound to sugars and other low molecular weight substances. Covalent bonds connect insoluble forms to the structural elements of the cell wall (5). All kinds of bioactivity have been shown to be antioxidant, antimutagenic, antifungal, and anti-inflammatory (7).

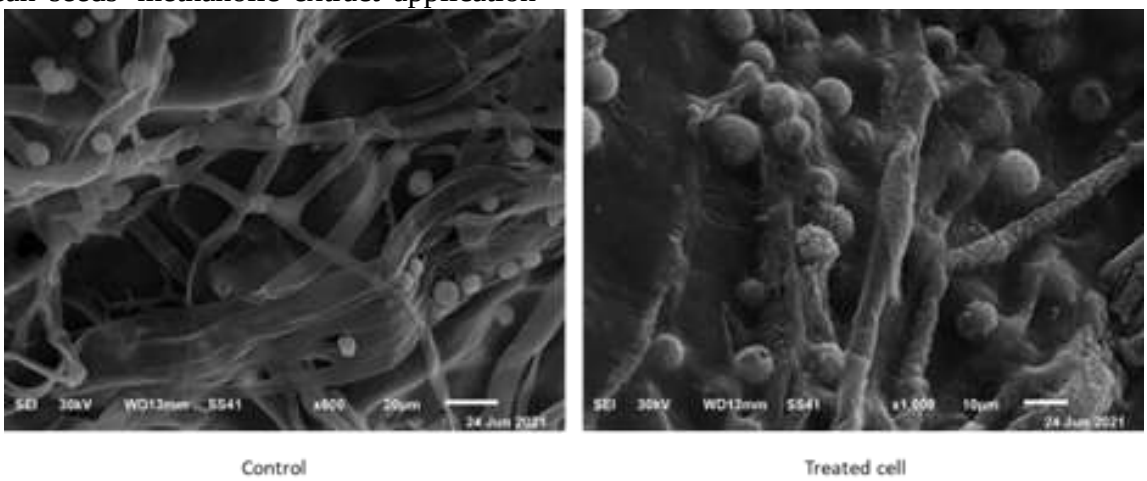

Figure 6. Scanning electron microscopy of Pythium spp treated with soybean seeds methnolic extract (100 µg/mL)

CONCLUSIONS

Considering the results, it is possible to employ the methanolic extract from soybean seeds, which is rich in phenolic chemicals, as an antioxidant, antibacterial, and antifungal agent. It functions well as a pure, natural product.

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