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Antioxidants and *Aspergillus niger* Antifungal Activity *in vitro* of Flavonoids Extract from Red-Top Leaves of *Cratoxylum prunifolium*

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The objective of the present study was to optimize flavonoids extraction process from red-top leaves following the Soxhlet method, which is capable of producing the highest flavonoid yield and assess antioxidant and *Aspergillus niger* antifungal activity of the extract. Besides, this research aims to establish a foundation for the use of these flavonoids instead of synthetic compounds as natural antioxidant for postharvest spoilage management of agricultural products. Optimization of flavonoids extraction process was conducted using response surface methodology with a central composite design. Included extraction factors in RSM are: 70-90 % ethanol solvent concentration; 30-70 v/w solvent/material ratio; 60-80 °C temperature and 30-90 min time. Antioxidant activity of the extract was assessed based on measurements of reducing power and 1.1-diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging activities. Then, *Aspergillus niger* antifungal activity of the extract was also assessed. The highest flavonoids content was at 6.96 % at following optimal extract conditions: 90 % ethanol concentration; ratio of solvent/material of 70 v/w; extraction temperature and time are 80 °C and 30 min, respectively. Antioxidant and *Aspergillus niger* antifungal activity of the flavonoids extract were higher than those of ascorbic acid (used as a standard). The highest reducing power was 1.0914 µg/mL obtained at 140 µL of the highest flavonoids contents treatment from extract process (compared with 0.6832 µg/mL ascorbic acid at same conditions) and 76.6 % DPPH activities (ascorbic acid 62.81 %). From this the highest flavonoids contents treatment, at 4.8 % flavonoids extract concentration (MIC = 4.8 %) for *Aspegillus niger* antifungal activity was 100 % (fungi without development or completely inhibited). Therefore, the flavonoids have been recommended for development of bio-compounds used in postharvest storage and medicine.

Keywords: Antifungal activity, Antioxidants activity, *Aspergillus niger*, Flavonoids, *Cratoxylum prunifolium*.

INTRODUCTION

Cratoxylum prunifolium, commonly referred to as red-top leaf plants, belong to the family of Hypericaceae. In Vietnam, red-top leaves plant consists of 5 species namely *Cratoxylum neriifolium, Cratoxylum prunifolium, Cratoxylum harmandii, Cratoxylum polyanthum, Cratoxylum formosum* and has primary geographical distributions on the highlands and midland north of Vietnam. This plants have been reported to have components of saponin (taraxeron), tannin and flavonoids (flavonols and catechins) [1]. Natural flavonoids source are commonly found in fruit, vegetables [2], grains, flowers, tea and red wine (catechins) [3]. The major component of flavonoid in red-top leaves is quercetin hyperosid. Species of *Aspergillus* often cause damage to grain crops due to mycotoxins contained in the mold [4,5]. In addition, the mold is also found to contain more than 300 fungal metabolites toxic to man and animals [6]. Leaves, flowers and extracts of this plant were used in the preparation of drugs and was reported to possess valuable pharmacological and biological activities such as natural antioxidant activities [7], antifungal and antibacterial activity [8-10] and therapeutic agents such as hepatoprotective, coronary heart disease prevention,

anticancer and anti-inflammatory activities [2,11], antidiabetic, antiaging and antiplatelet activities [12]. This natural antioxidant activities are much stronger than synthetic antioxidants like butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) [13], β-carotene, vitamin E and vitamin C [14,15]. Therefore, flavonoids from plants were widely isolated from green leafy vegetables such as amaranth, spinach, blackberry leaves, red raspberry leaves and strawberry leaves [16]. However, red-top leaves have not been the focus of any research. In addition, recent trends on antifungal activity investigation of flavonoids have been carried out on many species of fungi such as *Aspergillus niger* [17], *A. fumigatus* and *P. citrii* [18], *A. flavus* [19]. Therefore, in this study, red-top leaves were extracted and tested for their antioxidant and antifungal ability against *Aspergillus niger* to find out extraction which has the best biological activity. The research results could act as a precursor for the application of the plant in disease management in farming, safe storage of crops, new drug production and food additives.

EXPERIMENTAL

Preparation of leaves samples: Whole red-top leaves samples (*Cratoxylum prunifolium*) were collected from Cu Chi district, Vietnam and then transported to the physio-chemical laboratory belong to Hi-Tech Agricultural Development and Research Centre. The leaves samples, after undergoing preliminary treatment (removal damage leaves, wash away), were spread on trays with thickness 5 cm and desiccated at 50 °C to reach moisture of 12 %. Lastly, dried leaves samples were packed and stored at room temperature for subsequent experiments.

Optimization of flavonoids extraction process from red-top leaves: Extraction process were carried out follow by methods of Zekovíc *et al.* [20] and Cai *et al.* [21]. 100 g of dried red-top leaves were ground with ethanol at 70 to 90 % concentration, the ratio of solvent/meterials is 30:70 v/w, extract temperature from 60:80 °C and extract time 30:90 min. In the end of extract process, the solution was raw filtered to decouple 2 phases (liquid and rafinat phase). The liquid extract solution was determined for total flavonoids contents. Experiment was arranged in accordance with response surface design with CCD and was done in triplicate. Total flavonoids contents was determined by colorimetric method according to description by Marinova *et al.* [22] due to created colour with $AlCl_3$. Colour intensity increasing follow flavonoids content and test samples was compared with standard curve.

A 50 mg catechin (used as standard) was dissolved with 80 % ethanol into 50 mL measuring flask to obtain stock solution of 1 mg/mL. Then, stock solution was diluted to create a series of concentration of 20, 40, 60, 80 and 100 µg/mL. 1 mL of diluted solution was pipetted into 10 mL measuring flask and then added with 4 mL distilled water and 0.3 mL 5 % $NaNO_2$ and 0.5 mL the mixture of 5 % $AlCl_3$ and 96 % ethanol and 2 mL 1 M NaOH and shaken. After 20 min, this mixture was added with distilled water to the final volume of 10 mL and shaking and colorimetric at 415 nm by a spectrophotometer (UV-vis Specord 50 Analytik Jena, Germany). For survey samples: 1 mL extract solution from red-top leaves was pipetted into 10 mL measuring flask, the steps followed by was carried out according to standard curve. Total flavonoids was computed following the formula: $F(\%) = (a \times V/m) \times n \times 10^{-6} \times 100$ (where a is catechin (µg/mL) contents determined from standard curve; v is total extract volume (mL); m is samples weight (g); n is diluted coefficients).

Assay of biological activities of extract from red-top leaves

Reducing power assay: The highest total flavonoids was used to assay reducing power following the method of Oyaizu [23] and Barros *et al.* [24] with modifications. This extract solution was taken to a series of 20:140 µL into developmental tube and then added sodium phosphate buffer 0.2 M, pH 6.0 to reach final volume of 1.5 mL in each tube. The mixture was shaken and then added with 0.5 mL of 1 % potassium ferricyanide. This mixture was incubated at 50 °C for 20 min and 0.5 mL of 10 % trichloroacetic acid and 2 mL distilled water and 0.5 mL of 0.1 % ferric chloride ($FeCl_3$) was added to the mixture. The mixture was shaken to stable in 5 min and the absorbance was measured at 700 nm by a spectrophotometer. Ascorbic acid was used as the positive controls. Higher absorbance of the reaction mixture will indicate higher reducing power.

Free radical scavenger activities of DPPH (1,1-diphenyl-2-picrylhydrazyl) assay: 1,1-Diphenyl-2-picrylhydrazyl (DPPH) was assayed for the free-radical scavenging activity of the *Cratoxylum prunifolium* leaf extract according to the method of Oyaizu [23]. Extract solution containing the highest total flavonoids was taken to a series of 20:140 µL into developmental tube and then diluted with distilled water to reach final total volume of 3 mL in each tube. 1mL DPPH 0.2 mM solution (Sigma, USA) was then added. The solution was shaken and allowed to stabilize in the dark for 30 min. The absorbance of mixture was measured at wavelength 517 nm with a spectrophotometer. Ascorbic acid was used as positive controls and the control was prepared as above without any sample. A low absorbance of the reaction mixture indicated a high free-radical-scavenging activity. The scavenging activity was estimated based on the percentage of DPPH radical scavenged as the following equation:

$$\text{DPPH scavenging effect } (\%) = \frac{A_{control} - A_{sample}}{A_{control}} \times 100$$

Assay of *in vitro Aspergillus niger* antifungal activities of red-top leaves extraction: *Aspergillus niger* fungi was purchased from the Biotek-Nam Khoa Company and then cultured on potato dextrose agar (PDA) medium and then incubated aerobically at ambient temperature for 10 days before using. This fungi was used in test antifungal activity of *Cratoxylum prunifolium* leaf extract which has the highest total flavonoids contents *in vitro.*

Potato dextrose agar (PDA) media were ready autoclave and then cooled to 50 °C, dissolve extract solution into PDA media to reach the series of concentration (0, 1.2, 2.4, 3.6, 4.8 and 6 %) and poured into each petri dish and solidify. Then *Aspergillus niger* fungi were inoculated at the petri dish center and incubated at room temperature for 7 days [25]. PDA medium does not extract solution used as control. Experiment was done in triplicate, per replication 15 petri plates. After 7 days, growth

of mycelial on the petridish was measured in diameter. The antifungal activities of the extracts as percentage inhibition of mycelial growth was calculated following formula:

$$\text{Inhibition (\%)} = \left(1 - \frac{D_a}{D_b}\right) \times 100$$

where D_a = mycelial diameter growth in treatment dish; D_b = mycelial diameter growth in control dish). Additionally, minimize inhibit concentration (MIC) of extract was also determined (the lowest concentration of samples showing clear wells or with complete inhibition of fungi growth.

Statistical analysis: All data were analyzed by JMP 9.0 software. Significant differences between treatments were showed through Duncan test ($p < 0.05$).

RESULTS AND DISCUSSION

Optimization of flavonoids extract process from red-top leaves: Flavonoids are naturally phenolic compounds existing in most plants. They include flavonols, flavones, catechins, flavanones, anthocyanidins and isoflavonoids [26,27]. Flavonoids was widely used in foods (colour, taste, fat oxidation prevention, vitamins and enzymes protection) [28], especially in dietary sources [2]. Therefore, flavonoid from red-top leaves plants was extracted and determined. The total flavonoid contents extracted vary depending on solvent concentration, ratio of solvent/meterials, extract temperature and time and the results are summarized in Table-1. The total flavonoid contents varied in the range of 0.34:6.96 % and reached the highest point at following extract conditions: ethanol concentration of 90 %, 70 v/w the ratio of solvent/material, 80 °C extract temperature and 30 min extract time.

The results flavonoids contents obtained by the following regression equation: Y= 2.2621429 – 0.685556*extract time (30.90) + 0.723125*ethanol concentration*the ratio of solvent/materials – 0.538125*ethanol concentration* extract time. Response surfaces corresponding to each pair of elements are shown in Fig. 1, the response surface on the top side, *i.e.* the highest performance ranges.

The optimum condition predicted by JMP software was solvent concentration of 116.46 %; 83.15 v/w the ratio of solvent/material; 69.50 °C extract temperature and extract time of 161 min, corresponding with the predicted flavonoids contents of 1.38 %. Because the extract time is prolonged, flavonoids content obtained was low. However, in the process, flavonoids content was measured at the highest following conditions: ethanol concentration of 90 %, 70 v/w the ratio of solvent/material, 80 °C extract temperature and 30 min extract time. This produced the highest flavonoid contents of 6.96 %. Flavonoids contents of numerous plants have been extracted and determined in previous studies giving good results. According to Miean and Mohamed [29], the flavonoids was measured in leaves of onion (1497.5 mg/kg quercetin, 391.0 mg/kg luteolin and 832.0 mg/kg kaempferol), nect black tea (1491.0 mg/kg) and papaya shoots (1264.0 mg/kg). In addition, vegetables such as soybean sprout (78.5 mg/kg), red spinach (29.5 mg/kg) and kailan (14.5 mg/kg) are also found to yield high flavonoids contents in products. However, this contents was higher compared with our results.

TABLE-1
EFFECT OF SOLVENT CONCENTRATION, THE RATIO OF SOLVENT/METERIALS, EXTRACT TEMPERATURE AND TIME TO TOTAL FLAVONOIDS CONTENTS

Pattern	Ethanol concentration (%)	Ratio of ethanol/meterials (v/w)	Temperature (°C)	Time (min)	Flavonoid (%)
– – – –	70	30	60	30	3.20
– – – +	70	30	60	90	3.60
– – +–	70	30	80	30	3.93
– – ++	70	30	80	90	3.12
a000	70	50	70	60	2.10
– +– –	70	70	60	30	2.80
– +– +	70	70	60	90	2.61
– ++–	70	70	80	30	3.44
– +++	70	70	80	90	2.65
0a00	80	30	70	60	2.86
00a0	80	50	60	60	2.64
000a	80	50	70	30	3.00
0000	80	50	70	60	2.97
0000	80	50	70	60	2.97
000A	80	50	70	90	2.05
00A0	80	50	80	60	2.96
0A00	80	70	70	60	0.34
+– – –	90	30	60	30	2.42
+– – +	90	30	60	90	1.36
+– +–	90	30	80	30	2.71
+– ++	90	30	80	90	1.96
A000	90	50	70	60	2.11
++– –	90	70	60	30	5.97
++– +	90	70	60	90	2.24
+++–	90	70	80	30	6.96
++++	90	70	80	90	2.50

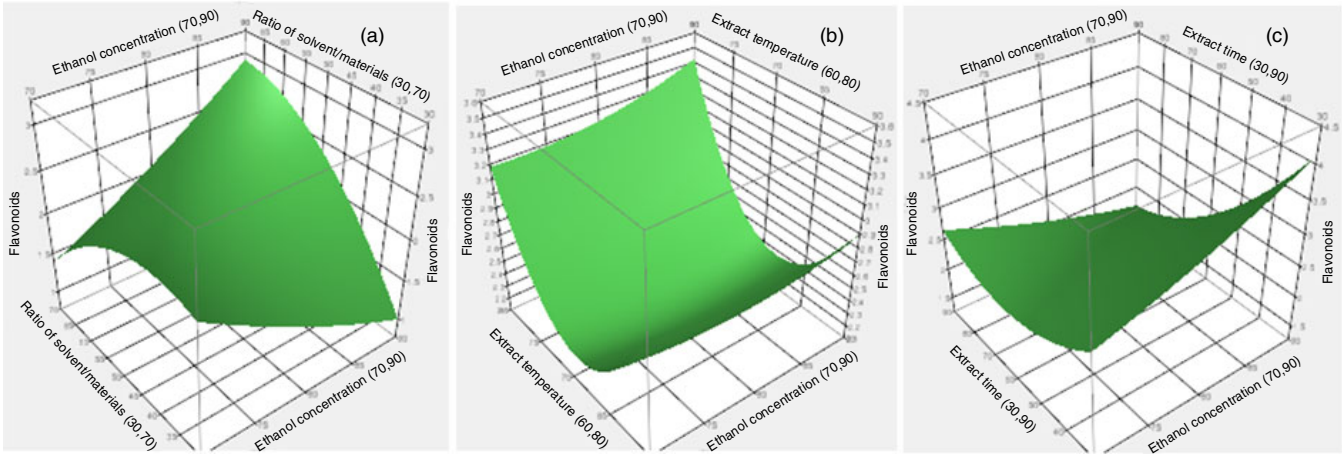

Fig. 1. Response surface showing the correlation between flavonoid content between: (a) ethanol concentration and the ratio of solvent/meterials; (b) ethanol concentration and extract temperature; (c) ethanol concentration and extract time

Biological activities of the extract from red-top leaves

Reducing power: The measurement of reducing power is the reductive ability of antioxidant. It was proved by the transformation of Fe(III) to Fe(II) in the presence of the sample extracts [30]. The extraction samples which had the highest total flavonoids in this study were used to assess for their antioxidant capacity. The antioxidant capacity of the extract from plant is affected by the extract composition and testing system and cannot be fully determined by a single method [31]. Meanwhile, reducing power was used to access antioxidant ability of extract red-top leaves *in vitro* condition affected by extract concentration and warelength absorbance. A higher absorbance indicates a higher reducing power [32]. The reducing power of red-top leaves extracts was found in all concentration used, when increasing extract concentrations by increasing value of reducing power (from 20 to 140 µL), but it was higher than with Ascorbic acid and results was showed in Fig. 2.

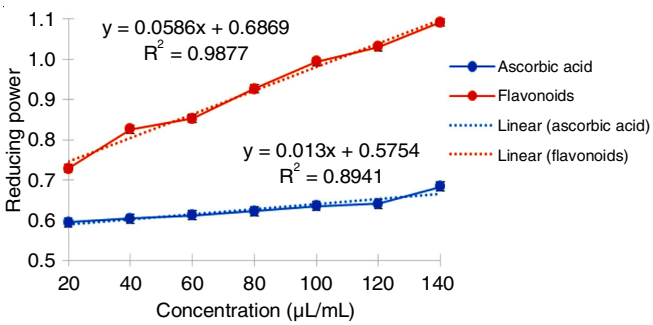

Fig. 2. Antioxidants activity of *Cratoxylum prunifolium* leaves extract by reducing power

The results of extract solution showed that reducing power peaked at 140 µL concentration and was significantly higher than that of ascorbic acid (positive control site) at same concentration. In different treatments, flavonoids treated extracts also showed higher reducing power in comparison to the control. This could be due to the creation of complexes such as oxychromon, oxycarbonyl or 3',4'-*ortho*-dioxyphenol when flavonoids reacts with ion metals, which is not possible in mixtures of ascorbic acid. Besides, higher absorbance is demonstrated at higher concentrations of extract. Free radical chain will lead reducing from the yellow Fe^{3+} to blue Fe^{2+} suggested the high total flavonoid contents [27]. Additionally, major components of flavonoid in red-top leaves are quercetin hyperosid, quercetin and their derivatives which are considered to be strong antioxidants. Their results was in accordance with those reported by Sakanashi *et al.* [33] and Braca *et al.* [34]. The properties of quercetin allow for inhibition of lipid peroxidation [35] and low-density lipoproteins (LDL) oxidation [36]. On the other hand, strong antioxidant potential of quercetin and their derivatives is due to their mechanisms [37-39].

Reducing power of extract obtained at difference concentration have been reported by previous study. According to Chang *et al.* [40], the crude extract had the highest reducing power at 2 mg/mL concentration. However, their results showed concentration of crude extract is lower and there is no report on the reducing power of red-top leaves extract was found out in previous studies. Meanwhile, significantly higher reducing power (1.47 ± 0.14 at 250 µg/mL) was evident in *Torilis leptophylla* fraction [32].

DPPH: 1,1-Diphenyl-2-picryl hydrazyl (DPPH) is a free radical generating compound using in checking the radical scavenging activity of extracts and the different type of antioxidant substances [41]. The colour of DPPH will change from violet to yellow when processed hydrogen capture or electron donation reducing [42,43]. A lower absorbance at 517 nm expressed higher radical-scavenging activity of the extract. According to Fig. 3, the DPPH radical-scavenging activity of red-top leaves extracts, ascorbic acid (used as standard substrate) were the ratio of the percentage increase parallel the increased concentrations.

Our finding have indicated that the highest DPPH radical-scavenging activity was obtained at 140 µL concentration (76.6 %). However, in comparison with positive control, but was still higher than ascorbic acid (62.81 %) at same concentration and difference between the treatments. The radical scavenging activity of total extract of *Cratoxylum prunifolium* leaves extract was even higher compared with *L. leucocephala* leaves extract (60.7 % for compound **3**) studied by Haggag *et al.* [44]. This is explained by the extract which has high total flavonoid contents parallel high scavenge DPPH radicals capacity. It

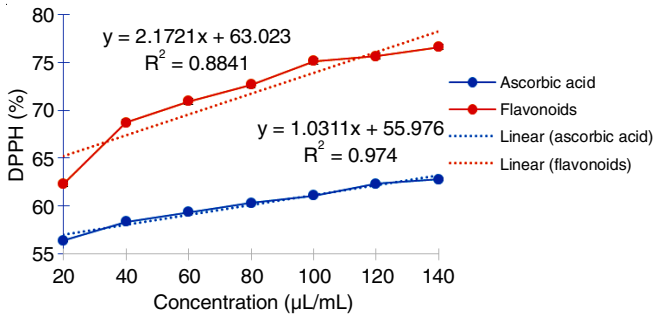

Fig. 3. Antioxidants activity of *Cratoxylum prunifolium* leaves extract by DPPH

depends on high hydrogen-donating resulting in high DPPH radical-scavenging activity [27,45]. Additionally, the differences could be explained by their structural conformations, distribution of hydroxyl groups and their position replacing other substitutions [46]. According to Zhao *et al.* [47], these polyphenols are more potent antioxidants than vitamins C and E.

Flavonoids are naturally compounds present in plants and are often used as a potential natural antioxidative [27] and anti-aging source. Therefore flavonoids from plants was extracted and used extensively from some previous study. Positive antioxidant effects on health stem from their ability to prevent damage from reactive oxygen species (ROS), prevent their generation [48] and help reducing the risk of diseases. Antioxidants are added in food for preventing or delaying the oxidation of food during storage and processing [49,50], due to free radicals formed using in the environmental as air, light and temperature [51].

***in vitro Aspergillus niger* antifungal activity of red-top leaves extraction:** Insects and microorganisms can cause pre and post harvest bio-deterioration and spoilage of agricultural product, grains, fruits and vegetables with losses of up to 100 %. Especially, species of *Aspergillus* fungi causing considerable loss in nutritional quality of grains and seed quality have been reported [52]. Several flavonoids from different plants have been extracted and used to inhibit fungal growth [18,53,54]. Meanwhile, *Cratoxylum prunifolium* leaf extract is also tested to access antifungal ability against *Aspergillus niger*. The results show that antifungal capability of *Cratoxylum prunifolium* leaf extract against *A. niger* (Fig. 4) progressively increased with increased concentration (diameter of fungi growth progressively decreased).

Percentage inhibition of *Aspergillus niger* fungi was increased from 0 to 100 % when increased crude extract concentration of red-top leaves from 0 to 4.8 %. In this study, PDA media containing 4.8 % red-top leaves extract concentration showed the highest percentage inhibition of fungi (100 %) compared with the control. Therefore, MIC of *Cratoxylum prunifolium* leaf extract against *Aspergillus niger* fungi was determined as 4.8 %. Strong antifungal potential of red-top leaves extract in this study might be due to the presence of flavonoid compounds such as saponin, tannin, isomangiferin, epigallocatechin gallate, gallic acid, especially quercetin hyperosid. Additionally, according to Dhiman *et al.* [55], their activity can be due to their complex ability with extracellular and soluble proteins or synergic effects of numerous flavonoid components present in crude extract. Final results are similar as reported by Clara *et al.* [17], high activity of compound quercetin-3-O-α-rhamnopyranosyl-(1‴→2″)-β-glucopyranoside and quercetin-3-O-α-rhamnopyranoside against *Aspergillus niger* (58.08 and 58.08 %) respectively with MIC of 2 mg/mL. Additionally, several flavonoids with antifungal activity isolated from different plants also gave results similar with our results. Further eicatechin isolated from *Azadirachta indica* leaves was found to inhibit the growth of *A. niger, A. fumigatus* and *P. citrii* [18]. Anthocyanidine isolated from *Bryophyllum pinnatum* was found to be active against the plant pathogen *A. niger* and the clinical fungus *Candida albicans* [56].

Conclusion

Crude extract from *Cratoxylum prunifolium* leaves was extracted and obtained the total flavonoid contents the highest 6.96 % at extract conditions:ethanol concentration is 90 %, 70 v/w the ratio of solvent/material, 80 °C extract temperature and 30 min extract time. Their extract solution has good antioxidants and *Aspergillus niger* antifungal capability at 140 µL concentration (76.6 % by DPPH radical-scavenging activity assay) and MIC = 4.8 %, respectively. Further research should be carried out to determine exactly adding some properties of extract and to test ability curative on human, adding in food or using to storage of grains and fruits.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this article.

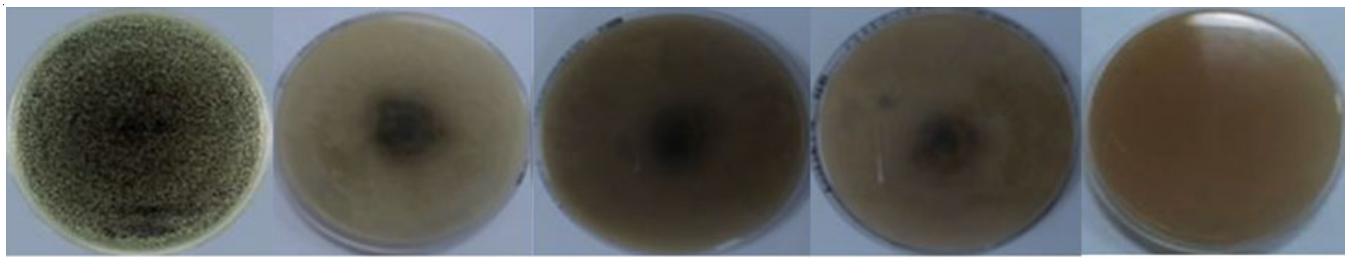

Fig. 4. Antifungal activity of *Cratoxylum prunifolium* leaves extract against *Aspergillus niger* at different concentration compared to control

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