Rice Husk Ash as an Adsorbent for Isolated the Celephthalides Compounds from Root of Apium Graveolens Linn for Inhibited Phytopathogenic Fungi in Plants

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***Abstract*—Apium graveolens Linn. is in Apiaceae family, the common name is A. graveolens or celeric. Chemical constituents of A. graveolens found sedanolide, celerin, celephthalide and flavonoids compounds which are repellent mosquito, antioxidant and antibacterial. This study used the root of A. graveolens were extracted by methanol. The crude methanol extracted isolated by the stationary phase of rice husk ash (RHA). Modified the powders of RHA by sieve sizes of Mesh 20, 40 and 100 gave three particle size are 660, 382 and 143 μm respectively, and cleaned by sodium hydroxide. The crude methanol was isolated by each of the particle size of RHA gave F1-F3 and were examined chemical constituents by thin layer chromatography. The fungicides used Rhizopus nigricans is a fungus commonly known as black bread. The orchid farms in Thailand found R. nigricans damaged leaves and roots of orchids. F1-F3 were tested by antifungal assay, as disc diffusion dilution, turbidity cell density is measurement by % transmitant at 625 nm by ultra-violet spectrometer and minimum fungicidal concentration (MFC). F1-F3 were inhibited fungicides in 68, 53 and 21 μg/ml, respectively. The aimed of this study used RHA from the rice-bran oil industry were absorbent for isolated the phytochemical compounds from A. graveolens which have the anti-fungicidal activity.**

***Index Terms*—Adsorbent, antifungal, celephthalide, rice husk ash, sieves.**

I. Introduction

Celery is scientific name *Apium graveolens* Linn., it belongs to the Apiaceae family. The native habitual of *A. graveolens*. is the lowland of Italy and now it is grown in everywhere in the world. The growth of seed generation within a two week period so cold and dry climate is suitable climate and are sown in march-April and seed, transplant in May and the crop ready to harvest in November [1]. The characteristic odor of clearly is due to a series of phthalide derivatives, sedanolide, senkyunolide-N, senkyunolide-J,4-hydroxymethyl-6-methoxy-2,-3-dihydro-1 H-indol -2-oland 3′-methoxy apiin [2], and they have bioactivities such as anti-inflammatory, antioxidant and cyclooxygenase and topoisonmerase inhibiteory [3]. The extraction of seed, leaves and stems gave polar compounds as furocoumarins (celerin, bergapten, apiumoside, apiumetin, apigravrin, osthenol, isopimpinellin, isoimperatorin [4] celereoside, and 5 and 8-hydroxymethoxy psoralen) [5], phenols (graveobioside A and B, apiin, apigenin, iso-quercitrin, tannins), alcohol and fatty acids (phytic acid) and glycosides [6]. The aerial part plant of *A. graveolens* were extracted with water gave the combined total phenolic and flavonoid using Folin-Ciocalteu method [7]. Apiin is a flavonoidglucoside, and it can acutes anti-inflammation [8]. A. graveolens is the medicinal herbal market which was observed by scientific studies and used as plantanthelmintic, antispasmodic, carminative, diuretic, emmenagogue, laxative, sedative, stimulant, and toxic [9]. Reported, *A. graveolens* was antifungal by the polar component of flavonoidglycosides and essential oils [10]. The objectives of the present paper are reduced and prevent the fungicides in orchid's farms in Thailand. Orchid's farms are in the area which were the wet and humidity which the fungicides were grown. The chemical drugs are used for anti-fungal as Flutolanil, AtpininA-5, Carboxin, Penthiopyrad, Boscalid, Fluopyram, Dinitrophenol -17 and Benzofurazan [11]. Rice husk ash, a residue of the rice barn oil industries, account for approximately 12% of the total weight of rice. It contains more than 60% amorphous silica, 10–40% carbon, and minor contents of other minerals [12]. This waste, very abundant in grain-producing regions , may be applied in the adsorption of different compounds, such as heavy metals in waste water (Ni^{2+}, Cu^{2+} and Zn^{2+} ions) [13].

The particle sizes of the powders of RHA were used sieve analysis by though brass sieve sizes of 20, 40 and 100 mesh. Crudes methanol extracted of *A. graveolens* were isolated by column chromatography (CC) used RHA 1 was absorbance and solvent system of ethylacetate: acetone: water (3:3:4) gave F1 and repeated with RHA2 and RHA3 absorbance gave F2 and F3, respectively. *A. graveolens* as phytochemical medicines for infections the fungicides and they are safety for plants and are generally to be used for farms. Anti-fungal assay used *Rhizopus nigricans* is a fungus commonly known as bread mold, and is the most common species of *Rhizopus genus*. It is found on spoiled food, in soils, and is dispersed in hot dry weather; the spores are allergy by chronic cough, dyspnea, chest tightness, chronic phlegm, snuffle, and allergic rhinitis [14].The research aimed to used crude extracted of *A. graveolens* of roots for inhibition *R.nigricans* which were fungicides in phytopathogenic fungi in the orchids farms. Antifungal methods were used disc diffusion dilution and measurement by % transmitting at 625 nm by spectrometer and were IC_{50} of the minimum fungicidal concentration (MFC) [15, 16]. In *vitro* anti-fungal activities were developed against for plant pathogenic strained of *R. nigricans* by using the roots

Manuscript received May 21, 2019; revised July 24, 2019.

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doi: 10.18178/ijcea.2019.10.5.756

extracted of *A. graveolens*.

II. Material And Methods

A. Plant Material

An a.graveolens part plant of roots was collected at Rajchabure province, Thailand in November 2017.

B. Extraction and Isolation

The dried roots part of *A. graveolens* was powdered in a blender 100g, it extracted individually with methanol. Crude extracted with methanol 10 g applied on the RHA1(200g) by CC, eluted with the solvent system of ethylacetate: acetone : water (3:3:4) gave F1 and solvents were removed by rotary evaporate at 50°C, repeated but changed the absorbent were RHA 2 and RHA 3 gave F2 and F3. Compounds 1 (scopoletin) [17] and 2 (Celephthalide) [18], were compared with literature. Compounds 1 and 2 elucidated by UV IR ^{1}H, ^{13}C-NMR and mass spectrometer. F1 was recrystallized with methanol and chloroform (1:9) gave Celephthalide ($C_{18}H_{24}O_8$) compound 2 and F2 was recrystallized with methanol gave compound 1.

Compound **1**: (6-methoxy-7-hydroxy-coumarin or scopoletin)**:** pale yellow needles ($CHCl_3$: MeOH; 9:1). mp 203-204°C. UV (MeOH)λ_{max} 254, 366 nm. IR ($CHCl_3$) ν_{max} 3400-3550 (OH), 1685 (C=O), 2998, 2938, 2856(CH), 1589, 1511 (C=C) cm^{-1}. APCI-MS 192.2008 $[M]^+$(calc. $C_{10}H_8O_4$192.1708). ^{1}H NMR ($CDCl_3$, 300 MHz)δ 7.61 (1H, d, *J* 9.5 Hz, H-4), 6.90 (1H, s, H-5),6.85 (1H, s, H-8), 6.28 (1H, d, *J* 9.5 Hz, H-3) and 3.85 (3H, s, 6-OCH_3). ^{13}C NMR ($CDCl_3$, 75 MHz) δ 161.5 (C2),150.2 (C6), 149.7 (C7), 144.0 (C8a), 143.4 (C4), 113.4 (C3), 111.5 (C4a) ,107.5 (C5),103.2 (C8),56.4(OCH_3).TLC chromatogram with methanol: chloroform: water (1:5:0.2) as a mobile phase revealed at R_f 0.70 and gave blue fluorescence under UV 366nm.

Compound **2**: (Celephthalide)**:** pale yellow amorphous powder (MeOH). mp 285-287°C. UV (MeOH)λ_{max} 305, 443 nm. IR (KBr) ν_{max} 3400-3550 (OH), 1685 (C=O), 2998, 2938, 2856(CH), 1589, 1511 (C=C) cm^{-1}. APCI-MS 369 $[M]^+$(calc. $C_{18}H_{24}O_8$=368.0128). ^{1}H NMR ($CDCl_3$-CD_3OD, 300 MHz)δ 5.67(1H, d, *J* 9.5 Hz, H-3), 7.4 (1H, d, *J* 9 Hz H-4),7.6 (1H, dd,*J* 1.5,7.5 H-5), 7.4 (1H, dd, *J* 1.5,7.5 H-6), and 1.9 (2H, m,H-1′). 1.7 (2H, m,H-2′) 4.0 (1H, m,H-3′). 1.32 (3H, m,H-4′), 4.85(1H, d, *J*8Hz, GlcH-1). ^{13}C NMR ($CDCl_3$-CD_3OD, 75 MHz) δ 169.5 (C1), 82.05(C3), 122.7 (C4), 134.0 (C5), 129.3(C6), 125.5 (C7), 126.5(C8), 112.2 (C9), 31.1(C1′), 32.4(C2′), 78(C3′), 23.0(C4′), 104.3(Glc1), 75.3(Glc2), 78.6(Glc3), 71.7(Glc4), 78.3(Glc5), 62.7(Glc6). TLC chromatogram with methanol:chloroform:water (1:5:0.2) as a mobile phase revealed at R_f 0.20 and gave blue fluorescence under UV 366nm.

C. Phytochemical Screening by TLC

F1 to F3 fractions are monitored the chemical constituent by Si gel TLC Kieselgel 60F_{254} (Merck). The solvent system was used chloroform: methanol: H_2O (7.5: 2.0: 0.5), TLC screening was examined the terpenoids by 10% H_2SO_4-ethanol spray reagent, flavonoids by NP/PEG (mixed between diphenylboric acid and polyethyleneglycol) spray reagent under UV 366 nm and phenolic compounds by 10% $FeCl_3$ in ethanol spray reagent. Authentic standard were stigmasterol, genistein and gallic acid.

D. Adsorbent and Its Characterization

RHA, obtained from the vegetable oil public company Limitted, Thak Sin, Bangkok. Thailand. The powders of RHA was sieved though brass sieve sizes of 20, 40 and 100 mesh to obtained the fineness three particle size are 660, 382 and 143 μm, were RHA1 RHA2 and RHA3, respectively.

E. DPPH Radical Assay

Determination of antioxidant activity using 2, 2-diphenyl-1- picrylhydrazyl (DPPH) radical scavenging method by prepared 0.2 mM of DPPH in MeOH. F1 was prepared of serial concentration as 10, 20, 30, 40, 50, 100, 250, 500 and 1000 ppm. Pipettes 1ml each of concentration of F1 and added 1ml of DPPH to each well. Mixtures were variously shaken and left for 30 min in the dark. Absorbance was measured at 515 nm using MeOH as blank. Percentage of radical scavenging was calculated using the equation: S(%) = A_0-A_s /A_0x100, which A_0 is the absorbance of the control (0.2mM of DPPH, 2 ml), and A_s is the absorbance of the tested sample. The half maximal inhibitory concentration (IC_{50}) value represented the concentration of the samples which have the antioxidant activity. Results were compared with of L-ascorbic acid. The results were expressed in percentage of inhibition as mean of three replicates. Repeated were testing with F2 and F3.

F. Antifungal Activities Assays

The agricultural pathogenic fungi were obtained from the culture at Apply of Micro- biology program at Suansunandha University. Cultures of *R. nigricans* was maintained on potato dextrose agar (PDA) and was stored at 5°C. *R. nigricans* was used in the experiments.

Potato dextrose agar (PDA) plates were prepared using 9 cm diameter plastic petri dishes. F1 was serial dilutions 100, 75, 50, 25, 12.5 μg/ml. PDA plates were containing 20 ml of agar and spread a suspension hyphal of *R. nigricans* 10 μl (10^6 CFU/ml) on the solid medium plate. PDA plate was tested for 5 serial dilutions of 100, 75, 50, 25, 12.5 μg/ml of F1 by pipetted 100 μl of sample on dises (5 mm diam).PDA plated treated with 100 μl of benomyl, prepared at a concentration of 1 mg/ml were used as positive control. The plates were incubated in 26°C. All tests were performed in triplicate. F1 was an inhibitory effect on mold growth in the disc diffusion assay by the concentration effect, and replicated with F2 and F3. The extension-diameter (mm) of hyphal from canters to sides of the dishes were measured for 7 days. The diameters of the inhibition zones were measured in millimeters. Growth inhibit one of treatment against control was calculated by percentage, using the follow formula: % Inhibition =(C-T)/Cx100 where C is an average of replicated of hyphal extension (mm) of controls and T is an average of 3 replicated of hyphal extension (mm) of plates treated with either crude extract dilutions. IC_{50} showed by the minimum fungal concentration (MFC) were measured by using microplate's reader. Prepared F1 was serial dilutions 100, 75, 50, 25, 12.5 μg/ml. Pipetted 100 μl of each of dilutions of the test sample was transferred into the wells of a

96-well plate, as well as the positive control (benomyl, 2mg/ml in DMSO)and blank (solvent) controls (DMSO and water). Pipetted 10µl (10^6 CFU/ml) in media of Muller Hinton broth of *R. nigricans* in 96 well plate. The plate was incubated for 24h, at 26°C for *R.nigricans*. Spectrophotometric measured data of the inhibitory by % transmittance at 625nm.The test was carried out in duplicate and the average % transmittance value was calculated. The relative inhibition (%) of the test sample was calculated by dividing the % transmittances value of the test sample by the average % transmittance of the solvent control.

III. Results and Discussion

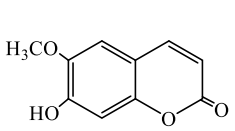
A. Phytochemical Scanning by TLC

The roots part plant of *A. graveolens* were isolated by absorbent of RHA 1-3 gave F1(4.2g), F2(5.6g) and F3(6.7g), respectively. F1-F3 were showed the phytochemical components on the TLC screening. The mobile phase of solvent system were $CHCl_3$: MeOH: H_2O (7.5: 2.0: 0.5). TLC showed the phytochemical components of terpenoids by 10% H_2SO_4 in ethanol using spray reagent and heat at 110°C were violet sport, NP/PEG spray reagent was activated flavonoids under uv 366 nm and phenolic compound were detected by 10% $FeCl_3$ in ethanol were dark spots. The authentic standard were stigmasterol, quacetin and gallic acid were reference. TLC screening the chemical constituents of *A.graveolens* in F1-F3 were summarized in Table I.

TABLE I: Components of Phytochemical of A.Graveolens in F1-F3 Detected by TLC Screening.

Isolation fractions	Weigh of dried crude (g)	% yields	Components on TLC screening
F1 : RHA1(660µm)	4.2	42	phenolics,celephthalide
F2 : RHA2(382µm)	5.6	56	flavonoidglycocides
F3 : RHA1(143µm)	6.7	67	essential oils, terpenoids. scopoletin, phenolicglycosides, flavonoid

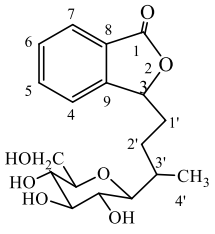
F1 was recrystallized with methanol gave Celephthalide (C18H24O8) compound 2 and F3 was recrystallized with methanol and chloroform (1:9) gave compound 1.



$C_{10}H_8O_4$ Mr 192

scopoletin

1



$C_{18}H_{24}O_8$ Mr 368

2

Fig. 1. Structure of compounds 1 and 2.

B. Antioxidant Activity

The polar solvent as H_2O and ethanol extracted the polar compounds of phytochemical constituents as flavonoid-glycosides terpenoidsglycoside and phenolic compounds in F1-F3. These results are in agreement of F1-F3 were tested antioxidant activity used DPPH reagent, and IC_{50} were 125.8, 48.8 and 75.9 µg/ml, respectively (Table II).

TABLE II: IC_{50} of inhibited DPPH Radical Scavenging of F1-F3 of *A. Graveolens.*

Isolated fraction	50% Inhibited of DPPH radical scavenging (µg/ml)			Average	SD	ID_{50}(µg/ml)
	1	2	3			
F1 : RHA1(660µm)	125.9	125.6	125.3	125.8	0.3	125.8±0.3
F2 : RHA1(382µm)	47.9	48.9	48.5	48.4	0.5	48.4±0.5
F3: RHA1(143µm)	74.9	76.1	75.6	75.5	0.6	75.5±0.6

C. Antifungal Activities

Previous reported, essential oils were isolated from seeds of *A. graveolens* and were tested anti- fungal[4]. Therefore, the studied were extracted essential oils and other phytochemical from leaves of *A. graveolens* , and were tested in anti-fungal assay. F1-F3 were effected of hyphal of fungal of *R.nigricans* by showed clear zone in disc diffusion dilution experiments. F1 F2 and F3 in concentration 12.5 µg/ml were inhibited clear zone 58 ±1, 54±1, and 51 ±1, respectively. Anti-fungal of F1-F3 were summarized in Table III.

TABLE III: Effective of Clear Zones of F1-F3 of Crude Extracted of *A. Graveolens*

Conc.(µg/ml)	Clear zons (mm)		
	F1	F2	F3
100	65±1	62±1	55±1
75	62±1	60±1	53±1
50	61±1	58±1	53±1
25	60±1	56±1	52±1
12.5	58±1	54±1	51±1

TABLE IV: IC50 of Inhibited the Minimum Fungicidol Concentration (MFC)on F1-F3 of *A. graveolens*

Extracted	Inhibitory the minimum fungicidol concentration(µg/ml)			Average	SD	ID_{50} (µg/ml)
	1	2	3			
F1: RHA1(660µm)	58	57.3	58.3	57.9	0.5	67.9±0.5
F2: RHA1(382µm)	40.1	40.3	40.1	40.2	0.1	53.2±0.1
F3: RHA1(143µm)	15.5	16.0	14.3	15.3	0.9	21.3 ±0.9

F1-F3 were tested for inhibited hyphal of *R.nigricans* in 96 well plate. F1-F3 were serial dilutions 100, 75, 50, 25, 12.5 μg/ml and added 10μl (10^6 CFU/ml) of hyphal of *R.nigricans*, incubated for 24h, at 26°C. Spectrophotometer showed inhibited fungal of F1-F3 by % transmittance at 625nm. F1-F3 measured data of the inhibitory the minimum fungicidol concentration (MFC) in IC_{50} were 67.9±0.5, 53.2±0.1 and 21.3±0.9, respectively, and summarized in Table IV.

IV. Conclusion

Rice husk is pressed for the rice barn oil which have many by-product of using of rice husk. So, the industries used by-product for firing and gave rice husk ashes. Reported used RHA for absorbent the chemical color or waste water. Causative of RHA have silica and carbon which can apply RHA as absorbance for separated in column chromatography technique for natural product. The studied used RHA were stationary phase for separated pure compounds from *A.graveolens* which is growth as backyard garden, and have eaten for vegetable or scatter over on food. They are growth everywhere in the world, and were the medicinal market. *A. graveolens* was considered good sources of natural compounds with the significant for antifungal. In vitro, F1-F3 were inhibited *R.nigricans*, the resulted that the crude extracted of *A. graveolens* can developing for inhibited phytopathogenic fungicides in their orchid farms in Thailand.

Acknowledgment

This work was generously supported by Suansunandha Rajabhat University, Bangkok, Thailand. I am grateful for Assoc. Prof. Weena Jiratchariyakul Department of Pharma-cog nosy, Faculty of Pharmacy, Mahidol University Bangkok Thailand for providing chemicals and advice for this study.

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