

Phytochemical Compounds in *Arundo donax* L. Rhizome and Antimicrobial Activities

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ABSTRACT

Introduction: The aerial part of *Arundo donax* L., giant reed, is a well-known fuel source used in many countries. **Methods:** Phytochemical compounds in *A. donax* L. rhizome, sequentially extracted with hexane (HEX), dichloromethane (DCM), ethyl acetate (EA), and methanol (MeOH), were identified using gas chromatography-mass spectrometry. Antimicrobial activities of the rhizome extracts were evaluated using disc diffusion assay against yeast (*Candida albicans*), and bacteria Gram-positive (*Staphylococcus aureus* ATCC 25923, *Bacillus cereus* ATCC11778, and *Bacillus subtilis* ATCC6633) and Gram-negative (*Escherichia coli* ATCC25922). **Results:** The detected phytochemicals were screened against WILEY07 library; 84 compounds matched with a similarity $\geq 90\%$. All the characterized compounds were grouped based on their functional group. The major phytochemicals in the HEX, DCM, and EA extracts belonged to sterol groups, while lipids, fatty acids, and related conjugates were the main components of the methanolic extract. The other characterized compounds were hydrocarbons, phenolics, terpenoids, xanthenes, and xanthone. Growth of *B. subtilis* was inhibited by the HEX, DCM, EA, and MeOH extracts, whereas *B. cereus* growth was inhibited only by the DCM and EA extracts. However, growth of *E. coli* and *C. albicans* could be not inhibited by *A. donax* L. rhizome extracts. Analysis of the compounds as well as their antibacterial activities via hierarchical clustering showed that hexadecanoic acid is the major compound influencing *B. subtilis* growth, while, *B. cereus* growth was affected by xanthone. **Conclusions:** Rhizomes of *A. donax* L. is one potential source of antimicrobial agents and further applied in medicinal uses.

Key words: Giant reed, Bacteria, GC-MS, HCA.

INTRODUCTION

Arundo donax L. (Poaceae) is a giant reed like grass with versatile economic (biofuel) and biomedical uses. The rhizome of this plant grows horizontally underground and has a storage function which has been the source material for pharmaceutical and biomedical utilizations. Acidic and neutral/alkaline fractions have been reported to inhibit *Prymnesium parvum*, ichthyotoxic golden alga.¹ In addition, traditional medicine has used *A. donax* L. as an emollient and a diuretic agent.^{2,3} Furthermore, basic biomedical research has used the rhizome from which purified n-acetyl-D-glucosamine was obtained. This phytochemical compound has been reported to exhibit anti-proliferation activity against a human cancer cell line.⁴

In view of these biomedical uses, investigation of phytochemical compounds in *A. donax* L. is a warranted procedure in order to establish the knowledge needed to further exploit this plant for pharmaceutical uses. A number of phytochemical compounds from *A. donax* L. have been extracted using various procedures as well as isolated/identified using chromatographic techniques. For example, n-acetyl-D-glucosamine, a specific lectin, has been isolated from *A. donax* L. rhizomes using affinity chromatography.⁴ The phytochemical compounds of *A. donax* stems were extracted with acetone and re-dissolved in chloroform before

fractionation.⁵ The analysis of stem extract by gas chromatography (GC) and gas chromatography-mass spectrometry (GC-MS) revealed compounds such as long-chain n-fatty acids, n-alkanes, n-aldehydes, n-alcohols, monoglycerides, free and esterified sterols, and terpenols. Recently, the non-structural carbohydrates in *A. donax* L. were identified and the results revealed that the contents affected the dry matter and water extracts, which further caused the differences in their biomass.⁶ However, the identification of phytochemical compounds in *A. donax* L. is still unclear with concerning to their types and contents. Therefore, in this study, the *A. donax* L. rhizomes extracted with various solvents were phytochemically characterized including the antimicrobial activities of their metabolites.

MATERIALS AND METHODS

Plant materials and extraction

Whole plants of *A. donax* L. during the flowering stage were collected in Thailand. The voucher specimen was prepared by Forest Herbarium (BKF) Department of National Parks, Wildlife and Plant Conservation, Ministry of Natural Resources and Environment to use as plant reference (Reference number: BKF NO. 19581). The voucher specimen compared with that in BKF database was identified as *A. donax* L. by Mr. Sukid Rueangruera, Forester Professional Academician of BKF. *A. donax* L.

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rhizomes were dried at 50°C before pulverizing. Sequential extraction based on the polarity index of various solvents used hexane (HEX), dichloromethane (DCM), ethyl acetate (EA), and methanol (MeOH), respectively. The rhizome was initially extracted by immersing 1-g powder with 10 ml HEX, followed by 15 min of sonication, then overnight maceration. Consequently, the residues were removed from the HEX extract by filtration through a membrane paper (Whatman® no. 1). The residue was consecutively extracted with DCM, EA, and MeOH, respectively. Then, the solvents were removed from all the extracts by vacuum evaporation, and the extracts were stored at -20°C for subsequent antimicrobial activity test and phytochemical analysis by GC-MS.

GC-MS analysis

GC-MS was used to characterize the phytochemical compounds which were dissolved in their extracted solvents for a final concentration of 10 mg/ml. Methyl heptadecanoate was used as an internal standard. The extracts were analyzed using an HP5-MS capillary-fused silica column (30 m length, 0.25 mm I.D., and 0.25 m film thickness), helium as a carrier gas at a flow rate of 1 ml/min, and 250°C injector temperature in splitless mode. The oven temperature condition was as follow; 40°C initiation, increasing 4°C/min to 300°C/min and increasing 5°C/min to 320°C/min for 10 min. The mass range of analyzed compounds detected at m/z 30–500 amu.⁷ Screening against the WILEY07 library enabled characterization of the phytochemical compounds from *A. donax* L., with similarity of at least 90% warranting further analysis.

Antimicrobial activity assay

The antimicrobial activities assay was performed by the disc diffusion method⁸ against yeast *C. albicans* and two types of bacteria: 1) Gram-positive (*S. aureus* ATCC 25923, *B. cereus* ATCC11778, and *B. subtilis* ATCC6633) and 2) Gram-negative (*E. coli* ATCC25922). Bacteria were cultured in Luria-Bertani broth at 37°C for 16–18 h; the broth was replaced with fresh Luria-Bertani broth and the culture continued for 2–3 h. *C. albicans* was cultured in yeast extract peptone dextrose (YPD) liquid medium at 30°C for 16–18 h; the medium was replaced with fresh YPD medium and the culture continued for 2–3 h. Each microbial inoculum was diluted in Mueller-Hinton (MH) broth to obtain 0.1 OD₆₀₀. Then, the pour plate technique was performed by mixing 1 ml of all microbial inocula with 9 ml of MH agar. All extracts were prepared by dissolving in 100% dimethyl sulfoxide at a concentration 100 µg/µl. The extracts (calculated as 2 mg/disc) were dropped on paper discs sized 6 mm in diameter and allowed to dry in a laminar flow cabinet; 100% dimethyl sulfoxide was used as a control. The discs with extracts were placed on microbial plates before overnight co-incubation at 37°C for all tested bacteria, and 30°C for *C. albicans*. Growth inhibition was detected measuring the clear zone.

Hierarchical clustering analysis (HCA)

The extracted phytochemical compounds with similarity values ≥ 90% and antimicrobial activity (only inhibited microbial strains) were grouped using HCA as previously reported by Sumner et al. (2003).⁹ The overall dataset was visually analyzed and defined by multiexperiment viewer (MeV), as Pearson correlation, using average linkage clustering.¹⁰ The relationship between detected compounds and antimicrobial activity was presented as a heat map pattern with the degree of difference indicated in a representative color.

RESULTS AND DISCUSSION

Phytochemical compounds in *A. donax* L. extract detected by GC-MS

Dried rhizomes were sequentially extracted with HEX, DCM, EA, and MeOH, respectively, as described in the experimental section.

All the four extracts were stored at -20°C before to analysis of the phytochemical compounds by GC-MS. The compounds were screened against the WILEY07 library; 84 phytochemicals were identified and the compounds with a matching-similarity ≥ 95% were presented in Table 1.

Methyl heptadecanoate was used as the internal standard for comparing the phytochemical contents in different extracts. Nineteen identical compounds were detected in the HEX extract, such as 2-benzoxazolone and heptadecane, which were not found in the other extracts. Five, 11, and seven specific compounds were detected only in DCM, EA, and MeOH extracts, respectively. Interestingly, two compounds, phenol, 3,5-bis(1,1-dimethylethyl)- and hexadecanoic acid (or palmitic acid), were detected in all the extracts. The most diverse identified compounds (49 compounds) were detected in the HEX extract, whereas fewer compounds were detected in the DCM (38 compounds), EA (34 compounds), and MeOH extracts (18 compounds). Use of various solvents for extraction resulted in different contents and types of compounds.

All detected phytochemicals in each extract were grouped, as shown in Figure 1, based on their functional groups as follows 1) lipids, fatty acid and conjugates 2) hydrocarbons 3) phenolic compounds 4) sterols 5) terpenoids 6) xanthenes and xanthenes, and 7) other compounds.

The phytochemical compounds in *A. donax* L. stems analyzed by GC-MS revealed that long-chain fatty acids, alkanes, aldehydes, alcohols, ketones, sterols and conjugates, terpenoids, monoglycerides, and glucosides were the major constituents in stem fibres.⁵ However, in this study, sterols were found to be the major constituents of the HEX, DCM, and EA extracts from *A. donax* L. with 52%, 42%, and 30% relative content, respectively. Although sterol was found in methanolic

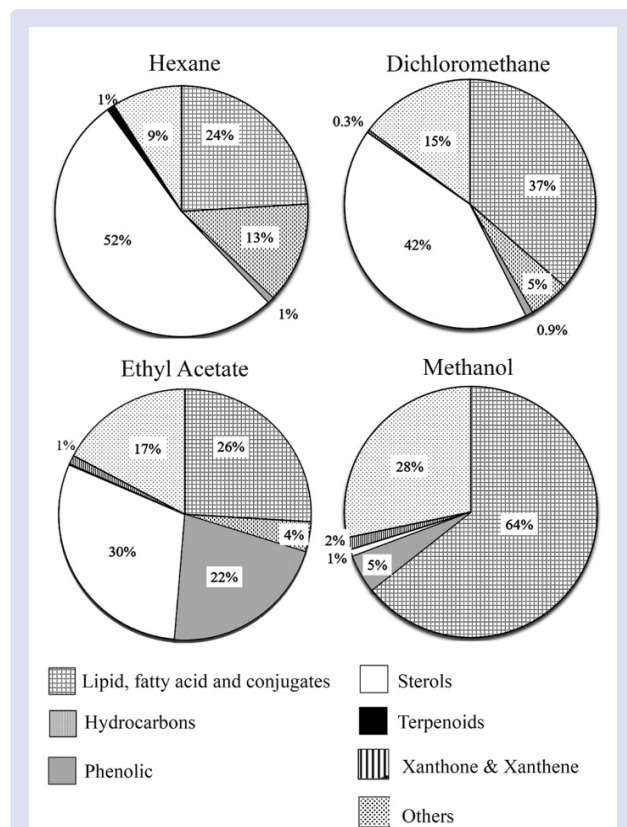


Figure 1: Phytochemical compounds (% proportion) detected in hexane, dichloromethane, ethyl acetate, and methanolic extracts of *A. donax* L. rhizomes.

Table 1: Phytochemical constituents in *A. donax* L. rhizome extracts characterized by screening against WILEY07 library after analysis by GC-MS.

RT (min)	Compound name	Match Similarity	Relative content (%)			
			Hex	DCM	EA	MeOH
7.43	2,3-Butanediol (CAS) Butane-2,3-diol	96	-	-	31.07	-
7.44	Heptane, 2,4-dimethyl-	97	2.48	3.78	-	-
15.57	Octane, 5-ethyl-2-methyl-	95	7.64	9.88	-	-
15.69	Undecane, 3,7-dimethyl-	95	-	-	0.81	-
17.07	Phenol, 2-methoxy-	98	-	-	4.67	1.30
17.28	Decane, 3,7-dimethyl-	96	2.12	-	-	-
20.67	1-Dodecene	98	1.41	-	1.44	-
23.31	Undecane, 4-ethyl-	95	1.60	1.51	-	-
23.90	Tetradecane	95	9.36	8.82	1.10	-
24.40	Dodecane, 4,6-dimethyl-	96	0.99	-	-	-
27.77	1-Pentadecene	97	0.59	2.52	-	-
31.00	Undecane, 3,8-dimethyl-	95	2.85	-	-	-
33.76	2(3H)-Benzoxazolone (CAS) 2-Benzoxazolone	95	90.95	-	-	-
34.20	1-Hexadecene	97	5.57	-	-	-
36.36	9H-Xanthene	95	-	-	3.94	1.44
39.92	3-Eicosene, (E)-	96	-	-	4.88	-
41.89	9-Octadecenoic acid (Z)-	97	-	9.07	-	-
42.21	Xanthone	96	-	4.25	2.50	0.52
44.68	Hexadecanoic acid	96	32.80	108.97	50.08	27.65
45.11	1-Docosene	97	5.67	-	-	-
45.98	Heptadecanoic acid, methyl ester	96	100.00	100.00	100.00	100.00
47.34	n-Heptadecanol-1	96	1.25	-	-	-
47.58	octadeca-9,12-dienoic acid methyl ester	96	-	1.55	1.72	0.93
48.28	Octadecanoic acid, methyl ester	95	0.64	-	-	-
48.73	9,12-Octadecadienoic acid (Z,Z)-	95	-	201.95	42.87	40.58
49.13	1-Octadecyne	96	6.25	-	5.59	-
53.26	Tetratetracontane	97	4.13	3.80	-	-
54.22	Tetracosane	98	5.55	4.77	-	-
54.54	Pentadecanoic acid, 2-hydroxy-1-(hydroxymethyl)ethyl ester	97	4.10	-	2.10	-
56.59	Hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl)ethyl ester	97	10.52	46.50	16.83	-
57.36	Di-n-octyl phthalate	96	-	-	-	0.95
58.16	1-TRICOSENE	96	-	-	0.70	-
60.11	Tetratriacontane	96	43.07	5.35	-	-
63.67	Heneicosane	97	29.12	7.65	-	-
66.93	Nonacosane	96	-	6.36	-	-
70.19	Stigmasta-5,22-dien-3-ol, (3.β.,22E)- (β-Stigmasterol)	96	142.86	111.12	25.81	-
71.30	Stigmast-5-en-3-ol, (3.β.,24S)- (β-Sitosterol)	95	292.90	249.88	90.91	-
71.54	Tetratetracontane	95	15.54	-	-	-

RT: retention time; HEX: hexane extract; DCM: dichloromethane extract; EA: ethyl acetate extract; MeOH: methanol extract.

extracts, it was not the main component (1%). Lipids, fatty acids, and conjugates accounted for the majority of the methanolic extract, with a content of 64%. The sterols or steroid alcohols, in *A. donax* L. included vitamin E, campesterol, 22,23-dihydrobrassicasterol, β-stigmasterol, β-sitosterol, and sitostenone. These sterols have been reported to have various activities, such as lowering plasma cholesterol,¹¹ anticancer effects against skin carcinoma,¹² and antidiabetic activities.¹³ Thus, *A. donax* L. rhizome maybe one important source of sterol compounds for further medicinal application. Phenolic compounds, such as phenol, 2-methoxy-; is another class that could be detected in all extracts; however, the EA extract presented the highest phenolic proportion at 21.5%.

Hydrocarbons were detected in all extracts, except the MeOH extract. Since the electrons of hydrocarbons are equally shared by the adjacent atoms between the bonds, this compound group is better extracted by a non-polar solvent or a solvent with low polar index. Hydrocarbon

compounds were detected in extracts derived from solvents with lower polarity index (P'), such as HEX ($P'=0.1$), DCM ($P'=3.1$), and EA ($P'=4.4$), but not in the MeOH ($P'=5.5$) extract.¹⁴ A low proportion of the terpenoid squalene was detected in the HEX (1%) and EA (0.1%) extracts. Squalene, a precursor of sterol production, was reported to have antioxidant and immune-boosting,¹⁵ and protective activities against ultraviolet-induced skin cancer.¹⁶ In the root part of several plants, genes involved in secondary metabolite production might be expressed at a low level, as revealed by the low content of artemisinin, a terpenoid with antimalarial properties, in the root part of *Artemisia annua*.¹⁷ Xanthone and xanthene were detected in the DCM (0.3%), EA (1%), and MeOH (2%) extracts. Xanthone could be found in various plant organs, such as fruit peel (e.g. mangosteen pericarp), bark, or root. It exhibited anticancer,¹⁸⁻¹⁹ immune modulation,²⁰ antioxidant,²¹ and antimicrobial activity.²² The analysis of *A. donax* L. extracts in this study revealed that rhizomes are an important source of valuable phytochemical metabolites, which could have medicinal

applications. All the four extracts were further analyzed for their effects on micro-organism growth, and the relationship between the detected phytochemicals and their anti-microbial activities was evaluated using HCA.

HCA of detected metabolites in *A. donax* L. and their antimicrobial activities

Antimicrobial activities of the four *A. donax* L. extracts including Hex, DCM, EA, and MeOH in crude forms were examined against the bacteria and yeast. Growth inhibition was determined based on clear zone after overnight co-incubation of the disc containing 2 mg of each *A. donax* L. extract with bacteria and yeast. The results showed that all four extracts inhibited the growth of *B. subtilis* as presented in Table 2; the DCM extract showed the highest activity (18 mm of inhibition zone). The HEX, EA, and MeOH extracts showed no significant activity against *B. subtilis* ($p \geq 0.01$). The growth of *B. cereus*, a human pathogenic bacterium, was inhibited by the DCM and EA extracts with 17.8 mm and 7.8 mm inhibition zone, respectively.

However, the HEX and MeOH extracts could not suppress its growth at this concentration. These results indicated that the DCM extract had the best antibacterial activity against the tested strains. Nevertheless, all four extracts could not inhibit *E. coli*, *S. aureus*, and *C. albicans* growth in this study. This first report proved that extraction of *A. donax* L. using different solvents resulted in differences in antibacterial activities. Moreover, *A. donax* L. has potential activities against *B. subtilis* and *B. cereus*. Since *B. cereus* is a Gram-positive human-pathogenic bacterium that causes foodborne diseases and some severe symptoms, such as vomiting and diarrhea,²³ the antibacterial activity of *A. donax* L. extracts, especially DCM extract, indicates that *A. donax* L. is a potential source of phytochemical compounds for antibiotic production.

Heat map visualization of antibacterial activities and relative phytochemical compounds was performed by HCA (Figure 2).

The results revealed that hexadecanoic acid had an important role in anti-*B. subtilis* activity as presented in the same cluster and pattern of heat map. Hexadecanoic acid, or palmitic acid, is a saturated fatty acid found in many organisms including plants. It was detected in all the extracts, with the DCM extract presenting the highest relative content (108.97%), while the MeOH extracts showed the lowest at 27.65%. This indicates that a higher hexadecanoic acid content would result in a higher anti-*B. subtilis* activity. In a further study, the anti-*B. subtilis* activity of *A. donax* L. extract could be predicted from this fatty acid content. Isolated n-hexadecanoic acid from *Canthium parviflorum* leaves also showed antimicrobial activities against *E. coli*, *S. aureus*, *B. subtilis*, and *C. albicans* at dose 100 and 150 μg .²⁴ According to the same pattern of expression in heat map, xanthone is the main compound which inhibited the growth of *B. cereus*. The DCM and EA extracts showed anti-*B. cereus* activities; xanthone was detected and identified in these two rhizome extracts with a content of 2.5-4.3%. Xanthone is a tricyclic aromatic compound, which could be found in the *A. donax* L. stem.⁵ In addition to anticancer, immune modulation, and antioxidant activities, xanthenes, the major active component derived from *Calophyllum brasiliense* Cambess. and *Mammea americana* L., showed antibacterial activities against *S. aureus* and *E. coli*.²⁵ The dose used in this study (2 mg extract/disc) was in the range similar to that used in previous studies, 1-2 mg/disc for *Andrographis paniculata*, *Morinda citrifolia*, *Piper sarmentosum*, and *Mitracarpus hirtus* L. extracts,^{8, 26, 27} and 5 mg/disc for *A. annua* extract.²⁸ Further studies on the investigation of bioactivity of *A. donax* L. rhizomes should focus on these two active compounds.

Table 2: Antimicrobial activities of hexane, dichloromethane, ethyl acetate, and methanolic extracts in *A. donax* L. extracts by disc diffusion test.

Tested microorganism	Inhibition zone (mm.) ¹²			
	Hex	DCM	EA	MeOH
<i>B. subtilis</i>	6.3 \pm 5.5 b ²¹	18.0 \pm 2.6 a	8.3 \pm 0.6 b	5.2 \pm 4.5 b
<i>B. cereus</i>	0.0 \pm 0.0 c	17.8 \pm 1.6 a	7.8 \pm 1.0 b	0.0 \pm 0.0 c

HEX: hexane extract; DCM: dichloromethane extract; EA: ethyl acetate extract; MeOH: methanol extract

¹²The inhibition zones were presented in mean \pm SD with 6 mm. diameter of tested disc.

²¹The alphabet represented the significant statistical differences after analysis by ANOVA, Duncan's test with $p \leq 0.01$.

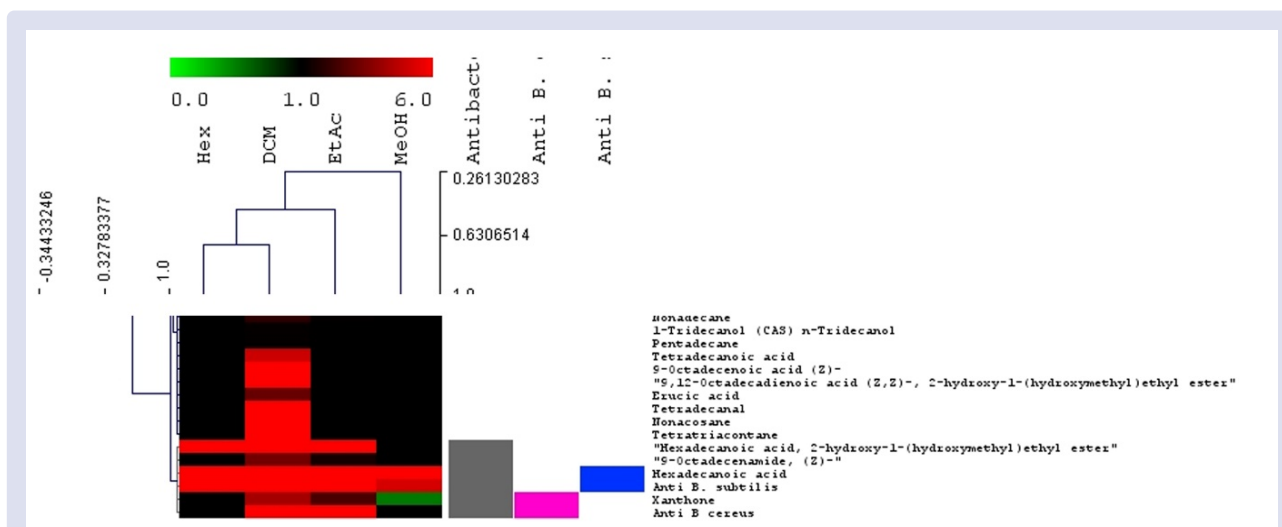


Figure 2: Heat map presented the relative different compounds of hexane (Hex), dichloromethane (DCM), ethyl acetate (EtAc), and methanolic (MeOH) extracts of *A. donax* L. rhizomes and anti-*B. cereus*/*B. subtilis* activities analyzed by HCA. The horizontal color scale above the heat map was ranged from green (low content) to red (high content). The vertical pink and blue bar represented the detected compounds relating with anti-*B. cereus* and *B. subtilis* activity, respectively.

CONCLUSIONS

The phytochemical compounds of *A. donax* L. extracted with HEX, DCM, EA, and MeOH, were characterized by GC-MS in this study. Grouping of the detected compounds revealed diverse phytochemicals in both quantity and quality, of which sterols were the major component of the HEX, DCM, and EA extracts. Nevertheless, lipids, fatty acids, and conjugates were the main components of the MeOH extract. Moreover, based on antibacterial activity, we recommend using DCM for extracting the phytochemicals from *A. donax* L. The study of *A. donax* L. rhizomes revealed that it has the potential to be used as a pharmaceutical source of phytochemical agents to treat diseases.

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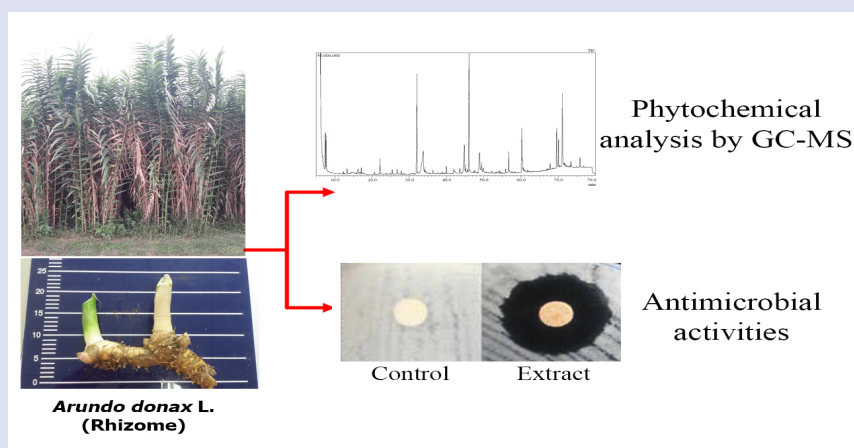
CONFLICTS OF INTEREST

None.

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GRAPHICAL ABSTRACT



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