Characterisation of allelochemical compounds signature in two mangrove forest species of *Rhizophora apiculata* and *Acrostichum aureum* and potential in suppressing weed growth

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**Abstract.** *Rhizophora apiculata* and *Acrostichum aureum* are a common mangrove species in Malaysia. Allelochemical interaction of the mangrove species was speculated to play an important role in dominance in the harsh mangrove environment. This study assessed the quantitative and qualitative determination for total phenolic content and individual phenolic compounds as allelochemical content for *R. apiculata* and *A. aureum* leaves as a potential allelopathic substance. The second objective is to investigate the allelopathic effect towards weed germination and growth through *in vitro* model system. Two types of maceration extraction comprising water extraction and sequential alkaline extract were used for allelochemical screening analysis. Both extractions were separated by ethyl acetate. *Acrostichum aureum* had the highest total phenolic content (1129.52 µg GAE/g DW) in comparison with *R. apiculata*. Meanwhile, the quantitative analysis by HPLC, revealed that different extraction detected different individual phenolic acid, Caffeic acid detected in both mangrove species in sequential alkaline extraction by *A. aureum*. The allelopathic effect of *Chloris barbata* for *A. aureum* extract indicated that during pre-emergent development, the suppression of weed growth was higher compared to post-emergent. Therefore, *A. aureum* may work as an allelochemical producer and can contribute to the establishment of pure colonies of *A. aureum* in the mangrove ecosystem.

1. **Introduction**

In the present day, a principal way of controlling weeds relies on herbicides [1] with the cost of using these products totalling in excess of 4.6 billion. About 3 million tonnes of herbicides are used to control weeds per year [2]. Many reports established on herbicides as alternative weed management but continuing use of herbicides in heavy doses of chemicals creates environmental pollution and increases the number of herbicide resistant weeds [2-5]. Approximately 99% of herbicides are released into the air, water and soil and only 1% reaches the targeted weed [6]. Such changes become crucial to preserve natural resources and product quality. Since triazine herbicides and photosynthesis inhibitors widely
implemented with inappropriate application of herbicides, it could contribute the accumulation of active compound in the soil and weed species [7].

Based on the current statistic of natural product-based practices, almost 70% are registered active pesticide ingredients have their origins in natural products research however only 7% of biochemical approved by the USEPA are bioherbicides [8]. Due to that, exploiting the allelopathy of plant-plant interaction as an alternative to control weed evokes a new prospect [9-10]. In the last decades, there has been an increasing approach known as allelopathic or weed suppressive as a green alternative that produce secondary metabolite termed allelochemical [11-15].

The term allelopathy was introduced by Austrian plant physiologist, Hans Molisch in 1937 who stated that allelopathy means biochemical interaction among higher plants, and between higher plants and microorganism, involving stimulatory and inhibitory actions [16-17]. In addition, by using the term allelopathy the plants that contribute allelochemicals are known as the ‘donor’ plant and the influenced plant recognized as the ‘target’ plant [18]. Allelopathy can be used to combat the challenges of environmental pollution and herbicide resistance development, and significantly for ecological, sustainable, and integrated weed management systems [19]. Thus, allelopathy has great potential in the production of phytotoxins to suppress another plant through natural interaction, through changes in the physiological process of plant growth. Allelochemicals were detected through species distribution, production of toxin, phytotoxicity effect and physical factors [20]. A natural component such as glucosinolate is considered the precursors of the allelochemical processing phytotoxicity as an effective way to suppress weeds growth [21]. In allelopathy context, phenolic compounds are described as simple aromatic phenols, hydroxyl and substituted benzoic acids and aldehydes, hydroxy and substituted cinnamic acid, coumarin, tannin and flavonoids [22]. Phenolic acid is a subclass of the larger phenolic category that occurs in food plants (fruit, vegetable, and grains) as ester or glycosides conjugated with other natural compounds such as flavonoids and alcohols and contain a phenolic ring and at least one organic carboxylic acid function [23]. Phenolic compounds are one major class of identified allelochemicals present in many submerged aquatic plants [24].

Weeds are often the biggest threat to organic crop production due to their dynamic community [25]. Weed is a serious pest of rice and causes annual worldwide rice yield loss by weed is 15-21% in which 42 different weeds species were identified from different categories of annual, perennial, grassy weeds, sedges and broadleaved weeds [26]. This study focuses on grassy weeds Chloris barbata found in abundance at selected study sites. Mangrove forest is known as harsh environment with complex abiotic factors, and different species composition which result in mangrove habitat complexity. However, the role of phytotoxic allelochemicals in mangrove community is still unknown, with only limited availability of information on relationship between allelopathy and mangrove succession [27].

This study aimed to investigate two mangroves species namely R. apiculata and A. aureum as potential allelopathic extracts. Three objectives were developed to achieve the research aim which was to (i) assess total phenolic content in R. apiculata and A. aureum qualitatively and to (ii) assess individual phenolic compounds quantitatively as allelochemical content using maceration extraction methods (water extraction and alkaline sequential extraction), and to (iii) investigate the allelopathic effect of R. apiculata and A. aureum extracts towards weed germination and growth through in vitro model system on Chloris barbata pre and post emergent.

2. Materials and methods

2.1. Plant material and sample preparation

The selection of R. apiculata and A. aureum was done randomly. R. apiculata (bakau minyak) and A. aureum (piai raya) were collected at Bagan Lalang, Selangor (2°36’38.6”N 101°41’06.8”E) in the mangrove forest. About 3 kg of fresh R. apiculata and A. aureum leaves were collected randomly, and later freeze-dried for three days and pulverized to powder, then stored in a 4°C chiller at Herbarium Laboratory, IIUM.
2.2. Plant maceration extraction

Two types of extraction methods were applied namely water extraction and sequential alkaline extraction.

**Water extraction.** 10 g of powdered freeze-dried material was mixed with 100 ml of distilled water and agitated on incubator shaker for 30 minutes at room temperature. Then, the sample was incubated in the oven at 60°C for 30 min before allowed to stand overnight in darkness at room temperature. The following day the sample was filtered before the sample was re-extracted with ethyl acetate as detailed by [28].

**Sequential alkaline extraction.** Sequential alkaline extraction is a method used to extract free and bound phenolic compounds from sodium hydroxide (NaOH) for 12h in the oven at 60°C. The alkaline extract was treated with hydrochloric acid (HCl) to reach pH 2, centrifuged and the supernatant extract was collected then re-extracted with ethyl acetate [29]. The final concentration was resuspended with methanol for further analysis by HPLC.

2.3. Determination of Total Phenolic Compound (TPC)

TPC determination using the Folin-Ciocalteau assay as reported by [30] was used with slight modification. Quantification was performed with hydrolyzed samples. Results were expressed as gallic acid equivalence (GAE) per gram dry weight sample using TECAN microplate reader.

2.4. Determination of Phenolic Acids Content with High-Performance Liquid Chromatography (HPLC)

HPLC analyses were conducted with the Agilent 1200 series (Agilent Technologies, Palo Alto, CA, USA) equipped with the binary pump, an autosampler and a diode array detector (DAD). The HPLC column was a reverse-phase Zorbax SB-C18 (Eclipse 100 × 2.1 mm, 1.8 µm). The temperature of the column was set at 25°C. For the analysis, two mobile phases were used consist of 1% formic acid in water/ acetonitrile 90:10 v/v (phase A) and acetonitrile (phase B). The solvent gradient used were developed as follows: 0-40% solvent B (0-20 min); 40-60% solvent B (20-25 min); 60-100% (25-25.1 min), 100% solvent B (25.1-35 min) and 100-0% solvent B (35-35.1 min) at a flow rate of 0.4 ml/min and with detection at 280 nm throughout the gradient [31]. The phenolic acids content was identified through their retention times and their UV spectra as compared to standards.

2.5. Determination of allelochemical effect towards weed germination

Two allelopathic species selection namely; R. apiculata and A. aureum extraction were explained in section 2.2.1. Seeds of the Chloris barbata (grass) were collected from a paddy field in Kepala Batas, Penang. The tissue culture processed was modified based on [32]. The concentration of the water extractions was 1g/ L prepared for the experiment [33] from low to high concentration (10 g, 20 g, 30 g, 40 g and 50 g). The experiment was observed from week 1 until week 4 and average of weeds growth were measured during pre-emergent and post-emergent of Chloris barbata.

2.6. Statistical analysis

Data were expressed as the mean ± standard deviation of triplicates solvents extraction for total phenolic compound (TPC) and phenolic acids content. One-way analysis of variance (ANOVA) with Tukey’s test was conducted using XLSTAT-Pro (2014) statistical software (Addinsoft, Paris, France).

3. Results

3.1. Analysis of total phenolic content in A. aureum and R. apiculata

These mangrove species showed significant differences in total phenolic content (TPC) (p < 0.0001; F:180.24; df: 10). A. aureum had the highest TPC between both mangrove species studied at 1129.52 (µg GAE/g DW) as presented in table 1.
Table 1. Total phenolic content (mg GAE/g DW) in two mangrove species.

| Allelopathic species | Total phenolic content (µg GAE/g DW) |
|----------------------|-------------------------------------|
| Acrostichum aureum   | 1129.52 ± 10.70                     |
| Rhizophora apiculata | 977.90 ± 2.13                       |

3.2. Analysis of phenolic acids content in *A. aureum* and *R. apiculata*

The phenolic acids analysis performed by HPLC system detected seven major phenolic acids: 4-hydroxybenzoic acid, caffeic acid, vanillic acid, trans-p-coumaric acid, ferulic acid, 3-coumaric acid and 2-coumaric acid. Analysis of variance established that both mangrove species exhibited highly significant differences in individual phenolic acids in water extraction (p < 0.0001). Both maceration extraction for phenolic acids content in two mangrove species as allelochemical is shown in figure 1. For water extraction, four individual phenolic acids were detected in *R. apiculata* (caffeic acid, vanillic acid, *trans*-p-coumaric acid and ferulic acid) whereas only three phenolic acids were detected in *A. aureum* (4-hydroxybenzoic acid, caffeic acid and 3-coumaric acid) (figure 1). The highest individual phenolic acid was ferulic acid at 1.56 µg/g DW in *R. apiculata*. Both mangrove species showed a different range of phenolic acids which was 0.02-1.56 µg/g DW in *R. apiculata* and 0.18-0.51 µg/g DW in *A. aureum*.

As for sequential alkaline extraction, the result showed that four individual phenolic acids were detected in *A. aureum* (4-hydroxybenzoic acid, vanillic acid, *trans*-p-coumaric acid and ferulic acid). However, only one phenolic acid was detected in *R. apiculata* (caffeic acid). The highest individual phenolic acid content was vanillic acid at 5.94 µg/g DW in *A. aureum*. In addition, the range of phenolic acid content for both mangrove species differed where 2.13-5.94 µg/g DW in *A. aureum* while 0.00-2.09 µg/g DW in *R. apiculata*.

Different extraction methods detected different individual phenolic acid. Sequential alkaline extraction produce the highest phenolic acids in both mangrove species compared to water extraction. *A. aureum* extract contained high amount of individual phenolic acid compared to *R. apiculata*.

![Water extraction graph with phenolic acid amounts](image-url)
3.3. **Analysis of allelopathic effect towards weed germination**

The purpose of this *in vitro* model system was to observe the propagation development of *C. barbata* seedling growth. Seeds of *C. barbata* were evaluated from day 0 until day 7 through *in vitro* model system cultured on MS basal medium as presented in Table 2. From the observation, the *C. barbata* seeds began to germinate and grow in day 2 whereas shoot length, shoot number, radicle length, radicle number and plant height also increased. Table 3 showed the effect of the allelochemical extracts from *A. aureum* on pre-emergence of *C. barbata* seed after 7 days. *C. barbata* seeds development showed a gradual stimulatory effect in germination rate, radicle length, radicle number, shoot length and shoot number in all concentrations. The inhibition of seedling development decreased from 95% to 20% as compared to control except for 50 g/L. Table 4 presented that the effect of an allelochemical extract of the *A. aureum* showed progressive inhibition effects on seedling growth during post-emergence of *C. barbata* as the concentration increased. The number of seedlings of *C. barbata* showed a reduction in growth from 10 g/L until 50 g/L concentrations in all allelochemical extracts as compared to control. In general, *A. aureum* allelochemical extract on pre-emergence of *C. barbata* caused a reduction in shoot length, radicle length, plant height and a number of the seedling. To sum up, *A. aureum* showed constituents of allelopathic activity which influenced the inhibitory effect of *C. barbata* allelochemical extracts as well as concentrations.

### Table 2. Seedling rate and growth index of *C. barbata*.

| Day  | Shoot height (mm) | No. of shoot | Length of radicle (mm) | No. of radicle | No. of seedling |
|------|-------------------|--------------|------------------------|----------------|----------------|
| Day 0| 0.0±0.0           | 0.0±0.0      | 0.0±0.0                | 0.0±0.0        | 0              |
| Day 1| 0.0±0.0           | 0.0±0.0      | 0.0±0.0                | 0.0±0.0        | 0              |
| Day 2| 1.0±0.0           | 1.0±0.0      | 1.0±0.0                | 1.0±0.0        | 1              |
| Day 3| 3.8±0.2           | 1.0±0.0      | 7.7±0.5                | 0.9±0.3        | 26             |
| Day 4| 5.6±0.2           | 1.0±0.0      | 11.7±0.4               | 1.0±0.0        | 36             |
| Day 5| 7.0±0.2           | 1.0±0.0      | 12.6±0.5               | 1.0±0.2        | 37             |
| Day 6| 7.7±0.3           | 1.0±0.2      | 13.3±0.3               | 1.0±0.0        | 32             |
| Day 7| 8.7±0.3           | 1.3±0.5      | 13.4±0.4               | 1.0±0.0        | 31             |
Table 3. Effect of allelochemical extracts of *A. aureum* on the pre-emergent of *C. barbata* seeds after day 7.

| Extract concentration (g/L) | Length of shoot (mm) | No. of shoot | Length of radicle (mm) | No. of radicle | No. of seedlings |
|----------------------------|----------------------|--------------|------------------------|---------------|-----------------|
| 0  (control)              | 9.9±0.4              | 1.4±0.5      | 10.9±0.4               | 1.0±0.0       | 25              |
| 10                         | 9.5±0.4              | 1.0±0.0      | 8.5±0.5                | 1.0±0.0       | 2               |
| 20                         | 14.5±0.1             | 1.0±0.0      | 8.5±0.8                | 1.0±0.0       | 2               |
| 30                         | 5.0±0.0              | 1.0±0.0      | 5.0±0.6                | 1.0±0.0       | 3               |
| 40                         | 9.0±0.4              | 1.0±0.0      | 8.5±0.9                | 1.0±0.0       | 2               |
| 50                         | 10.0±0.0             | 1.0±0.0      | 7.0±0.0                | 1.0±0.0       | 1               |

Table 4. Effect of allelochemical extracts of *A. aureum* on the post-emergent of *C. barbata* seeds after day week 4

| Extract concentration on (g/L) | Length of shoot (mm) | No. of shoot | Length of radicle (mm) | No. of radicle | No. of seedlings |
|-------------------------------|----------------------|--------------|------------------------|---------------|-----------------|
| 0  (control)                  | 12.0±0.3             | 1.0±0.0      | 15.6±0.6               | 1.0±0.0       | 25              |
| 10                            | 10.4±0.3             | 1.0±0.0      | 14.1±0.3               | 1.0±0.0       | 14              |
| 20                            | 12.3±0.2             | 1.1±0.3      | 14.0±0.4               | 1.0±0.0       | 9               |
| 30                            | 13.8±0.4             | 1.4±0.5      | 16.8±0.1               | 1.0±0.0       | 5               |
| 40                            | 14.5±0.2             | 1.2±0.4      | 19.2±0.4               | 1.0±0.0       | 6               |
| 50                            | 12.8±0.1             | 3.6±0.5      | 35.6±4.9               | 1.0±0.0       | 5               |

4. Discussion

Phenolic compounds is produced by the plant in response to environmental stress in the mangrove ecosystem. Fern species *A. aureum* is a mangrove species with tolerance to high salinity and environmental stress, hence high TPC was detected from this fern [34]. High salinity in the soil causes difficulty to absorb sufficient amounts of water, thus decreasing cell osmotic potential [35-36]. Furthermore, plants respond towards environmental condition by developing sophisticated mechanism such as cell regulation [37]. As stated by [38], allelopathic plants depend on resistance mechanism leading to detoxication: conjugation, sequestration and oxidation which may switch on partially to oxidative stress [34]. In this research sequential alkaline extract was found to be a potential resource of the bioactive allelochemical compound compared to water extraction. This finding was supported by [38] who mentioned that sequential alkaline extraction is a method used to extract free and bound phenolic compounds from plant materials. Phenolics in plant species largely appear to be linked with plant cell wall polysaccharides by both hydrophilic and hydrophobic bonds in which enzymes might disintegrate the phenolic-cell wall matrix bonds and enhance phenolic extraction as well as important for the stability of the phenolics in the extract [39].

The allelochemical extracts from *A. aureum* affected germination and growth differently. The result indicated that most of the target species demonstrated a gradual reduction of growth development at different inhibitions as concentrations increased. According to [40], plants have different abilities to tolerate allelochemicals to reduce the uptake of the allelochemicals also to detoxify the allelochemicals from the target site. According to [41], the exact mechanism involved on inhibitory effect was caused by allelochemicals interfering with the physiological and biochemical process in target species.

5. Conclusion

In conclusion, by considering the result from allelopathic treatment through the *in vitro* model system, the possible allelopathic mode of action and mechanism of the plant extracts that contained water-soluble (aqueous) phytotoxic constituents, as a potential natural herbicide was capable of interfering directly on germination and growth of other species especially weed. *A. aureum* and *R. apiculata* may produce phytotoxicity known as allelochemical to develop their community through regulatory,
constitutive and inductive mechanism towards the environment as a physical defense system. In other words, it could be incorporated as a natural herbicide for weed management control as an organic tool in horticulture as well as landscape management.

6. References

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