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Research

Antioxidant and Antibacterial Activity of Different Solvent Extracts of Leaves and Stem of *Alyxia reinwardtii* Blume

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ABSTRACT

Alyxia reinwardtii Blume (Pulasari) Apocynaceae is being widely used as a traditional medicine in treating various illnesses due to its phenolic, coumarins, lignans, iridoid glycosides alkaloids and flavonoids content. These compounds in *A. reinwardtii* were reported to be useful as medicinal and agricultural potentials. Hence, *A. reinwardtii* meets the rationale for antioxidant and antibacterial studies to replace synthetic substances using different solvents on the leaves and stem of *A. reinwardtii* using cold maceration. The Folin-Ciocalteu reagent assay was used to estimate the phenolic content of extracts. The total flavonoid content was determined using aluminum chloride. The antioxidant capacity of the samples was evaluated by 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical and Ferric Reducing Antioxidant Power (FRAP) assay. Disc Diffusion Assay (DDA), Minimum Inhibitory Concentration (MIC), and Minimum Bactericidal Concentration (MBC) were conducted to evaluate the antibacterial activity of *Escherichia coli* and *Bacillus cereus* growth. Methanol was determined as the most effective solvent due to the highest crude yield obtained, (19.47 ± 2.80 %). Acetone showed the highest TPC content (170.44 ± 10.99 mg GAE/g) while hexane showed the highest TFC content (2957 ± 91.24 mg QE/g). Moreover, for DPPH, methanol inhibited the highest antioxidant (75.81 ± 12.62 %) and hexane for FRAP assay (2278.89 ± 69.47 mg AAE/g). Hexane extract is potentially effective with variable efficiency with DDA (11.17 ± 4.48 mm) while methanol extracts with MIC (0.63 µg/mL) and MBC (2.50 µg/mL) against *E. coli*. The results indicated that methanolic and hexane extract using cold maceration showed optimum antioxidant and antibacterial activity. The present study advocates that *A. reinwardtii* was a source of substantial antioxidant and antibacterial agents for potential pharmaceutical applications.

Key words: Apocynaceae, DPPH, extraction, flavonoid, FRAP, medicinal plant

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INTRODUCTION

Oxidative stress plays an important role in human diseases. The phrases "free radicals" and "antioxidants" are widely known for the health-conscious individual. Active oxygen and free radicals are causing tissue harm leading to various diseases including cardiovascular disorders, aging, cancer, and neurodegenerative sickness (Pham-Huy *et al*., 2008). In recent years, natural antioxidants have been used as an alternative to replace synthetic drugs using medicinal plants (Ortega-Ramirez *et al*., 2014). Reactive oxygen species (ROS) including singlet oxygen, superoxide ion, hydroxyl ion, and hydrogen peroxide are highly reactive and toxic molecules that cause various human diseases such as cancer, atherosclerosis, diabetic mellitus, hypertension, and aging (Nadia & Rachid, 2013). Hence, combating such a situation has been so far dependent on natural antioxidants because of their ability to scavenge free radicals to donate hydrogen atoms or electrons (Anokwuru *et al*., 2011). Plant includes considerable extents of phytochemicals antioxidants such as ascorbic acid, phenolic compounds including flavonoids, phenolic acids, and volatile compounds to prevent oxidation biomolecules (Hadadi *et al*., 2020).

Moreover, pathogenic microorganisms are also infectious disease-causing serious illnesses to public health in both developing and developed countries. Microbes nowadays are resistant to drugs and cause infectious diseases to get worse (Anwar *et al*., 2015). On the other hand, researchers have also reported the emergence of multi-drug resistant (MDR) bacteria that caused treatment failure in many infectious diseases (Mohamed *et al*., 2013; Nadia & Rachid, 2013). MDR bacterial infections often lead to morbidity and mortality in humans (Marasini *et al*., 2015). Due to the increase in resistance to antibiotics and these negative effects, there is an urge to develop new and effective antimicrobial agents against microorganisms (Saiah *et al*., 2016). Hence, plant extracts are used to replace synthetic drugs as antimicrobials based on substances that kill or inhibit the growth of causative pathogens (Mahasneh & Al-Hussaini, 2011).

Generally, medicinal plants contain phytochemicals that play vital roles in determining the pharmacological properties existing in plants especially *Alyxia reinwardtii* Blume (Pulasari) which is a plant belonging to the family Apocynaceae shown in Figure 1. It is considered one of the potential Malaysian herbal products because of its bioconstituents such as; phenolic, coumarins, lignans, iridoid glycosides, alkaloids, and flavonoids content (Sugijanto *et al*., 2009). This plant contains three classes of secondary metabolites which were identified as coumarin and its derivatives, trimeric-iridoid diglucoside, and iridolactone (Peng-ngummuang *et al*., 2015). Reportedly, eight compounds have been isolated from stems of this plant namely, (1) coumarin, (2) 3-hydroxycoumarin, (3) 6-hydroxycoumarin, (4) 8-hydroxycoumarin, (5) scopoletin, (6) (+)-pinoresinol, (7) zhebeiresinol and (8) *p*-hydroxybenzoic acid as shown in Figure 2 (Rattanapan *et al*., 2012). The high content of 3-hydroxycoumarin derivatives in *A*. *reinwardtii* stem bark exhibits antioxidative activities by scavenging free radicals and inhibiting human low-density lipoprotein peroxidation (Nugroho *et al*., 2016). According to Topçu *et al*. (1990), two iridolactone was isolated namely alyxialactone and 4-epi-alyxialactone from the leaves of *A*. *reinwardtii* as shown in Figure 3. Although *A*. *reinwardtii* is widely used in traditional Thai medicine its pharmacognostic specification has yet to be established. Hence, it is very important to provide preliminary data on the biological activity of *Alyxia reinwardtii*.

Fig. 1. *Alyxia reinwardtii*, A. Plant with young leaves; B. Herbarium specimen; C. inflorescence; D. Unripe fruits; E. Ripe fruits

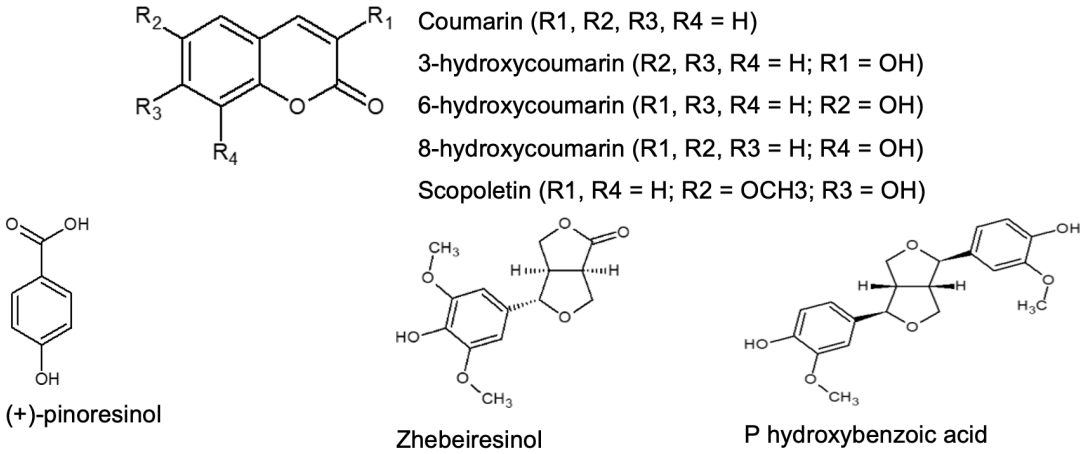

Fig. 2. Compounds isolated from *Alyxia reinwardtii* stems

Alyxialactone 4-epi-alyxialactone

Fig. 3. Compounds isolated from *A*lyxia *reinwardtii* leaves

Moreover, the need for widely usable and easily available natural antioxidant and antibacterial continue to grow. Hence, it is important to evaluate the antioxidant and antibacterial activity of this plant as it contains bioactive compounds (Anwar *et al*., 2015). The most common and simplest method of obtaining bioactive compounds from plant material is extraction. As phenolic compounds are dependent on the polarity of the solvents used, selecting the best solvent affects the quality and quantity of extracted phenolic compounds (Sepahpour *et al*., 2018). Therefore, this study proposed the most efficient solvent for the extraction of crude yield that produces optimum phenolic, flavonoid, antioxidant, and antibacterial activities on the stem and leaves of *A*. *reinwardtii* to provide preliminary data on its biological activity.

MATERIALS AND METHODS

Chemicals

The chemicals used in the study include absolute methanol, acetone, and hexane, sterile distilled water, Sodium bicarbonate, Folin-Ciocalteu reagent, Gallic acid, 5% Sodium nitrate, 10% Aluminium chloride, 1M Sodium hydroxide, Quercetin, 2,2-diphenyl-1-pycrylhydrazyl (DPPH), Ascorbic acid, Acetate buffer (pH 3.6), 2,4,6-tripyridyltriazine (TPTZ) solution, Nutrient broth, Mueller-Hinton Agar (MHA), Mueller-Hinton Broth (MHB), Ciproflaxin, *Dimethyl sulfoxide* (DMSO).

Preparation of crude extract

The whole plant *A. reinwardtii* was collected from Fraser Hill, Pahang, Malaysia, and a voucher specimen (MK884, 29.03.2019) was deposited at the Department of Biology, UPM. Plant parts were air-dried at 27 °C for 4 weeks on a plank board after being spread evenly. The dried stems and leaves were crushed and ground using a crusher and mill separately. The powdered form sample was kept separately in nylon pouches and kept in a freezer before extraction (Justine *et al*., 2019).

Cold maceration

Triplicates of 10 g of stems and leaves powder samples were soaked in 100 mL of 4 different solvents, respectively. Solvents that were used include absolute methanol, hexane, acetone, and sterile distilled water. All the extracts were shaken at 150 rpm in an incubator shaker continuously for 24 h at 25 °C(Obeidat *et al*., 2012).

Determination of extract yield

Stems and leaves liquid extracts obtained were separated from solid residue by vacuum filtration through Whatman No. 1 filter paper. Extracts were concentrated using a rotary evaporator and dried in a cooled vacuum oven at 40 °C until a constant mass was obtained before being weighed using an electronic balance. Meanwhile, for *aqueous* filtrates, lyophilized using a freeze-dryer till the dry powder is obtained and weighed (Adam *et al*., 2019). The weight of crude yield obtained by simple calculation as below,

$$\text{Extract Yield (\%)} = \frac{(\text{Mass of vial + extract, g}) - (\text{Mass of vial, g})}{10\ \text{g (DW)}} \times 100\ \%$$ -Equation 1

Total phenolic content

About 200 µg of crude sample and 1.5 mL of sodium bicarbonate were added with 1.5 mL

Folin-Ciocalteu reagent and incubated for 2 hr in a dark place. A spectrometer was used to measure the absorbance at 750 nm. Test samples were run together with gallic acid in the concentration ranging from 0 to 200 µg/mL. Standard curve of Gallic acid solution with the equation y= 0.0057x + 0.0025 where R^2 = 0.9929 and the results were expressed as mg Gallic Acid Equivalent (GAE) per g of dry weight (DW) (Hossain *et al*., 2021).

Total flavonoid content

Total flavonoid content will be determined using the method proposed by (Hossain *et al*., 2021). 1 mL of extract, 4 mL of distilled water, and 0.3 mL of 5% sodium nitrate solution were mixed. 0.3 mL of 10% aluminum chloride, 2 mL of 1 M sodium hydroxide, and 2.4 mL of water were added to the mixture and left to incubate for 15 min. Absorbance was measured using a spectrophotometer at 510 nm. Test samples were run with standard quercetin with 25 mg of quercetin in 50 mL of ethanol in the concentration ranging from 0 to 100 µg/mL. Measurements were plotted into a standard curve of Quercetin with the equation y = 0.0002x + 0.0286 where R^2 = 0.9985 and the results were expressed as mg Quercetin Equivalent (QE) per g of dry weight (DW).

Antioxidant capacity evaluation

DPPH radical scavenging activity

A method performed by (Abu Bakar *et al*., 2015). 2,2-diphenyl-1-pycrylhydrazyl (DPPH) was used as a free radical model to measure the antioxidant activity of the extract. DPPH solution (4 mg of DPPH in 100 mL of methanol) and crude extract (1 mg in 1 mL methanol) were prepared in a 96-well plate using 2 - the 2-fold dilution method. Ascorbic acid is used as the standard. The absorbance was read at 517 nm after incubating in the dark for 30 min at room temperature. The test was conducted three times. Radical scavenging activity was calculated using the equation:

$$\text{Scavenging effect (\%)} = \left[\frac{A_{\text{blank(517nm)}} - A_{\text{sample(517nm)}}}{A_{\text{blank(517nm)}}}\right] \times 100\ \% \qquad \text{- Equation 2}$$

A blank is the absorbance of DPPH and A sample is the absorbance of the sample with DPPH. Each sample was analyzed in triplicates.

Ferric Reducing Antioxidant Potential (FRAP) assay

This method was done according to (Ghasemzadeh *et al*., 2015). Stock solutions of 10 mL of acetate buffer with pH 3.6, 1 mL TPTZ (2,4,6-tripyridyltriazine) solution in HCL, and 1 mL of $FeCl_3$ solution were prepared. Crude extracts (100 µL) and deionized water (300 µL) were added to 3 mL of FRAP solution. The solution was mixed and incubated for 30 min in a water bath at 37 °C. Ascorbic acid was used as a positive control in this study. The absorbance of the resultant solution was measured at 593 nm using a spectrophotometer with acetate buffer as blank. A standard curve was prepared using different concentration values of $FeSO_4 \cdot 7H_2O$ with the equation y = 0.0003x + 0.098 (R^2 = 0.9961). The FRAP results were expressed as milligram Ascorbic Acid Equivalent (AAE) per g dry weight (DW).

Antibacterial activity

Disc Diffusion Assay (DDA)

This test was done according to the method described by (Inglin *et al*., 2015). Gram-positive bacteria strain, *Bacillus cereus,* and gram-negative bacteria strain, *Escherichia coli* were used to test the antibacterial properties of the crude. Bacteria were inoculated in nutrient broth and incubated for 24 h at 37 °C. A sterile cotton swab was used to spread the bacteria on Mueller-Hinton Agar (MHA). The sample disc (6 mm) was soaked in the sample-prepared MHA plate. It was then incubated at 37 °C for 24 h. Ciprofloxacin and DMSO were used as positive and negative controls respectively. The inhibition zone was observed and measured.

Minimum Inhibitory Concentration (MIC)

This test was conducted on a sterile 96-well microtitre plate by microdilution method described by Balouiri *et al*. (2016). A stock solution of crude extract (100 µL) was prepared by serial dilution in Muller-Hinton Broth (MHB) on a microlitre plate from columns 3-12. Microbial suspension (100 µL) was added to each well except for column 1 which contains broth only while column 2 was filled with bacteria and broth only (negative control). DMSO was used as a positive control in this experiment. The plate was left to incubate at 37 °C for 24 h. The lowest concentration of the extracts with no colony was observed after the incubation period.

Minimum Bactericidal Concentration (MBC)

A small amount (10 µL) of the sample was taken from the microplates pipetted on MBA plates. The plates were then incubated at 37 °C for 24 h. The MBC was determined as the lowest concentration of extract that inhibits 99.9% of bacteria. All the samples/ extracts were triplicated.

Statistical analysis

Results were expressed as mean ± SD of triplicates and data were analyzed using SPSS version 25. Pearson Correlation and ANOVA were carried out to test the relationship and difference between tests, respectively.

RESULTS AND DISCUSSION

Extract yields

According to Figure 4, methanolic extract showed the highest yield on leaf extracts (19.47 ± 2.80 %). The efficiency or optimization of crude yield is affected by a few conditions such as extraction method, temperature, extraction time, the composition of phytochemicals, and the solvent used (Hien *et al*., 2019). Alcohol is used because of its neutrality and extracts products obtained were compatible with other products. Polar solvents were one of the best solvents used because they can extract more active compounds from the plant (Sankeshwari *et al*., 2018). Some of the solvents that have low viscosity and density were very helpful because can diffuse easily into the pores of the plant material to extract bioactive compounds. In most studies, highly polar solvents were more efficient in extraction because the plant contained more polar compounds that are soluble such as in water, methanol, and ethanol (Hien *et al*., 2019). However, differences in the polarity of solvents also affect the bioactive compounds that will be extracted from the plants. According to the polarity of solvents, water (1.000) should show a higher extract yield compared to methanol (0.762), somehow, the results were the opposite. This shows that the chemical compounds of *A*. *reinwardtii* are more soluble in alcohol compared to water.

More so, according to the result obtained, leaves show higher extract yield compared to stem. Phenolic compounds were synthesized by pentose phosphate pathway, shikimic acid pathway, and aromatic amino acid through phenylpropanoid metabolization (Lin *et al*., 2016). Phenolic compounds are produced through photosynthesis to protect the plant against wounding, infection, and exposure to UV radiation, or as a defense mechanism (Cosmo *et al*., 2020). The shikimic pathway in plants is localized in the chloroplast which is found in leaves. This explains that more phenolic compounds are produced in leaves showing a higher extract yield compared to stem.

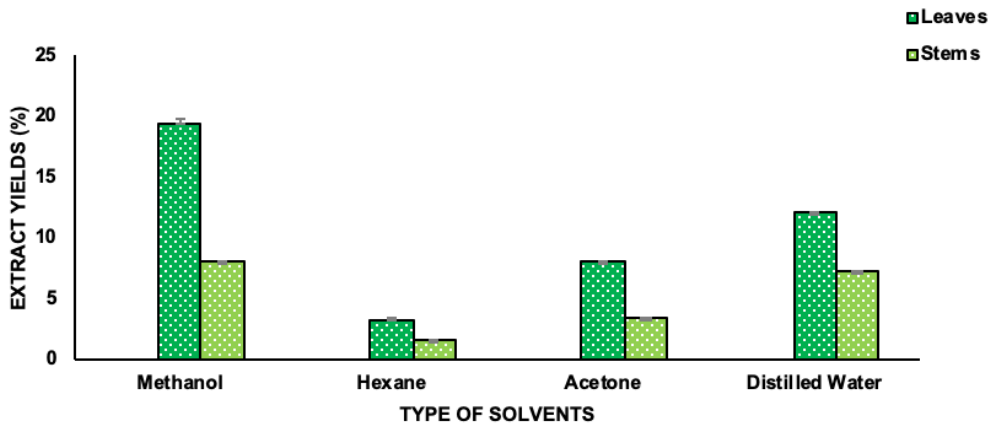

Fig. 4. Effect of solvents on extraction yields of *Alyxia reinwardtii* leaves and stems crude extracts

Total Phenolic Content (TPC)

The highest TPC was recovered with acetone on stem extracts (170.44 ± 10.99 mg GAE/g) as shown in Figure 5. Phenolic content increases as the polarity of the solvent increases, which supports the result shown above. Somehow, distilled water showed low phenolic content even though the polarity was the highest compared to methanol and acetone. This is because TPC was found to be decreased with the increased amount of water content in the aqueous solvent. This happens because the compounds were more soluble in absolute methanol, acetone, and ethanol (Do *et al*., 2014). For example, extracts of *Bauhinia vahlii* showed the highest polyphenolic content in methanol followed by acetone, hot water, and chloroform (Zlotek *et al*., 2016). Different solvents are used to extract different compounds such as acetone for proanthocyanidins and tannins extraction; ethanol for flavonoids and their glycosides, catechols, and tannins; while methanol for phenolic acids and catechin (Tan *et al*.,

2013). Since methanol and acetone showed higher phenolic content, hence extracted compounds could be phenolic acids, catechin, proanthocyanidins, and tannins.

Meanwhile, for the plant part, leaves extract of methanol and distilled water using cold maceration showed higher phenolic content than the stem. As the phenolic compounds are synthesized in chloroplast through the shikimate pathway, leaves contain high phenolic compounds (Iloki-Assanga *et al*., 2015). According to (Rafat *et al*., 2010), the methanolic extract of *Andrographis paniculate* showed higher phenolic compounds in leaves than stems which is the same relation to the result. However, due to differential gene expression, different plant part synthesizes and accumulate different compounds (Santo-Sánchez *et al*., 2019). For hexane and acetone crude, stem extracts showed higher TPC content compared to leaves. Polyphenol synthesized depends on plant species, type of organs, and growth stage (Stankovic *et al*., 2011). This is because, during the flowering stage, phenolic compounds were higher in leaves than stem. TPC decreases 2 times lower during the non-flowering stage compared to leaves collected at the flowering stage except for the stem which remains with the same amount of phenolic in both stages (Feduraev *et al*., 2019). Supporting the result shown for Total Phenolic Content (TPC).

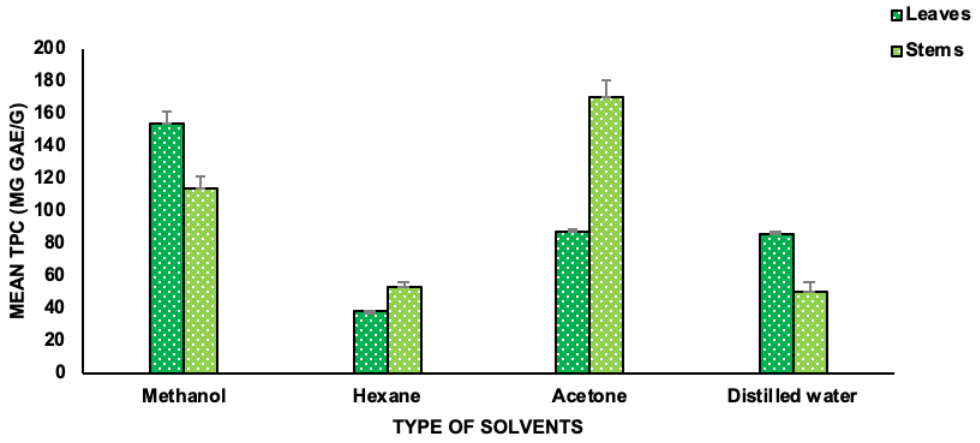

Fig. 5. Effect of solvents on Total Phenolic Content (TPC) of *Alyxia reinwardtii* leaves and stems crude extracts

Total Flavonoid Content (TFC)

Hexane showed the highest TFC on leave extracts (2957 ± 91.24 mg QE/g) (Figure 6). This is because different polarities of compounds are soluble in different solvents which affects the extraction of flavonoids (Ngo *et al*., 2017). Flavonoids contain hydroxyl groups that affect the biological activity of plants due to their structure and substitution (Dirar *et al*., 2018). Flavonoids can be highly polar or less polar as they vary in polarity. A less polar solvent is used to extract aglycones while a more polar solvent is used to extract glycosides and anthocyanin (Routray & Orsat, 2012). However, in this study, non-polar solvents showed higher flavonoid content, maybe because the compounds extracted are non-polar such as aglycones. Meanwhile, distilled water extract showed no TFC content. This is because several studies showed that flavonoids are best extracted using organic solvent due to the polarity of flavonoids (Chaves *et al*., 2020). Hence, organic solvents such as hexane, acetone, and methanol are more efficient in extracting flavonoids compared to distilled water in this study.

Consequently, both plant parts showed flavonoid content using the organic solvents. Flavonoids are produced to protect plants from biotic and abiotic stresses, as well as UV protection (Mathesius, 2018). Flavonoids are produced in the cytosol of the cell and are held in the endoplasmic reticulum membrane which is later collected in different places including the vacuole (Samanta *et al*., 2011). A vacuole is a cell that is found in the leaves of plants where biosynthesis of flavonoids occurs. Hence this may be a reason why more flavonoids are extracted from this plant part compared to the stem. However, for acetone extraction, the stem showed higher TFC than the leaves. According to (Panche *et al*., 2016), flavonoids are also found in bark, stem, root, and flower. Since leaves are attached to the stem at the areas called nodes, flavonoids can also be found in the stem of *A. reinwardtii.*

Scavenging activity on 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical

The highest DPPH activity was showed by methanol on leave extract (75.81 ± 12.62 %) as shown in Figure 7. Phenolic compounds are vital antioxidative plant components that are found well correlated in plant's antioxidant activity. It is the ability to scavenge the reactive oxygen species by donating a hydrogen atom or by single-electron transfer (Banjarnahor & Artanti, 2014). Flavonoid is one of the compounds that consist of hydroxyl functional groups, configuration, and substitution that affect some mechanisms in antioxidant capacity (Kumar & Pandey, 2013). Distinctive chemical

structures such as ortho-dihydroxy arrangement in the B ring, C2-C3 unsaturated bond combined with C-4 carbonyl group in the C skeleton, and O-methylation are required for efficient radical scavenging (Procházková *et al*., 2011).

Since, plant parts contain both phenolics and flavonoid contents, that contributes to antioxidant activity in *A. reinwardtii.* Thus, leaves and stems showed almost a similar result. A few other criteria that affect antioxidant activity are the type of solvent used, extraction method, and conditions that include temperature and time (Do *et al*., 2014). According to the result obtained, absolute methanol and acetone, as well as distilled water, showed high antioxidant activity. Some Bangladeshi legumes of acetone and methanol extracts showed high antioxidant activity (Hossain *et al*., 2021). According to (Boeing *et al*., 2014), methanol and water are considered some of the efficient solvents for extraction due to the better solvation of antioxidant compounds. This may be due to interactions (hydrogen bonds) between polar sites of the antioxidant molecules and solvent. The polar phase of these extracts contributes to the inhibition of free radicals through sample electron transfer and proton transfer (Herrera-Pool *et al*., 2021). High polar solvent proves to be better for inhibition of DPPH radicals in this study.

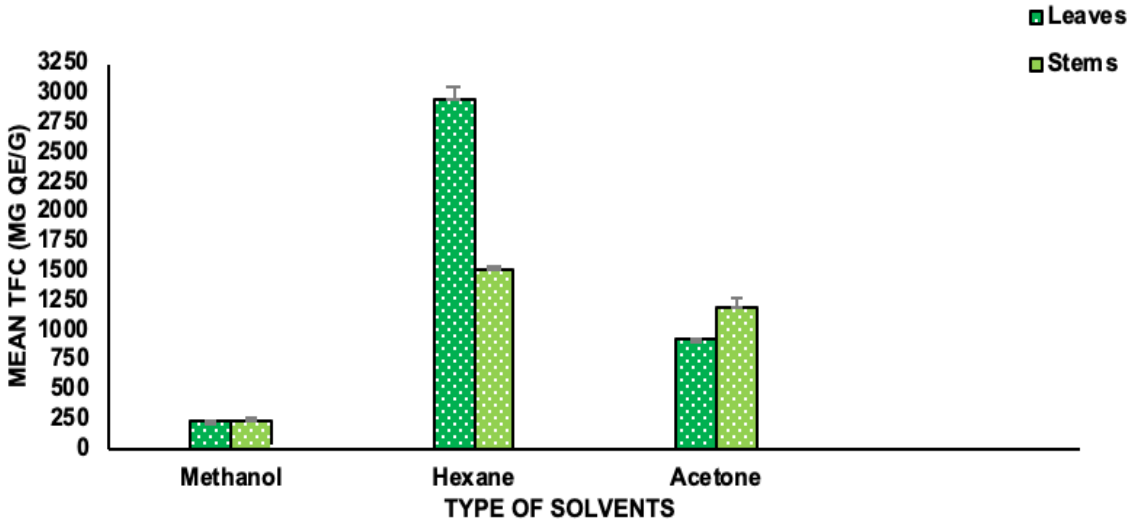

Fig. 6. Effect of solvents on total flavonoid content (TFC) of *Alyxia reinwardtii* leaves and stems crude extracts.

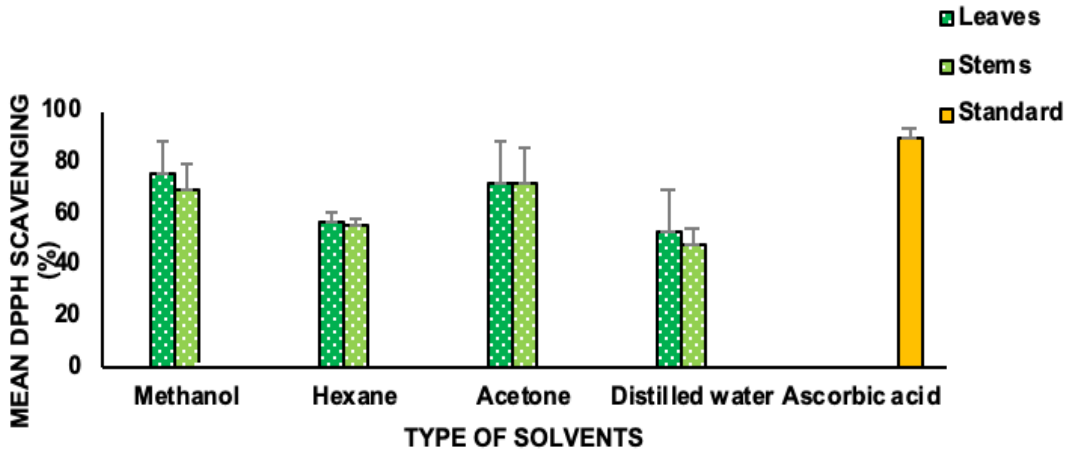

Fig.7. Effect of solvents on 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging (%) of *Alyxia reinwardtii* leaves and stems crude extracts.

Ferric Reducing Antioxidant Power (FRAP) activity

The effect of solvents on ferric-reducing antioxidant power activity is shown in Figure 8. Hexane extract showed the highest FRAP value on leaves extract (2278.89 ± 69.47 mg AAE/g). Phenolic compounds are responsible for antioxidant activity in FRAP assay due to their capability to scavenge free radicals by donating their electrons or H (Aryal *et al*., 2019). The degree of hydroxylation in these compounds plays a major role in redox potential (Kumar & Pandey, 2013). A few aspects affect the FRAP value such as solvent polarity, vapor pressure, and viscosity, the type of antioxidant compound extracted differs as well (Shahidi & Zhong, 2015). Supporting the result where hexane shows the highest FRAP value because of its low viscosity (0.330 Pa-s) compared to methanol (0.797 Pa-s) and distilled water (1.002 Pa-s). Low-viscosity solvents help to diffuse bioactive constituents from extracts better because of low density and high diffusivity (Wijekoon *et al*., 2011). Distilled water and methanol extract from *A*. *reinwardtii* showed no FRAP value in this study. According to Njoya (2021), phenolic structures with an ortho-dihydroxy moiety such as hydroxytyrosol could chelate Fe^{2+} whereas the tyrosol that has only one OH does not show that ability to reduce Fe^{3+} to Fe^{2+}. Hence, the extracted compounds by these solvents might contain one OH group in its structure which has no reducing power. Hence, this proves that hexane extracts showed the highest FRAP value from *A*. *reinwardtii*.

Correlation between extract yield, phenolic compounds, and antioxidant activities

The correlation study analysis of *Alyxia reinwardtii* plant part and solvent crude extracts is presented in Table 1 and Table 2, respectively. A strong correlation coefficient is shown between TFC and FRAP in leaves (r=0.940). High flavonoid content in leaves may contribute to the high FRAP potential as compared to other phenolic compounds. These free radicals are generated naturally in living cells during metabolism processes (Choudhary & Swarnkar, 2011). This result is supported by Wang *et al*. (2012), showing strong correlation between antioxidant activity and flavonoid compounds in 42 blueberries tested. A strong correlation is shown in the table below for the solvent system between DPPH with extract yield (r=0.971), DPPH with FRAP (r=-0.991), and FRAP with extract yield (r=-0.971). Plant phenolic compounds show a huge impact on the biological properties of plants such as antioxidant properties as it appears to correlate between both (Zlotek *et al*., 2016). More extract yields were accumulated in high extract yield hence this indicates higher phenolic compound and strong antioxidant activity. According to (Anwar *et al*., 2013), there is a direct correlation between phenolic compound, extract yield, and antioxidant activity which is by the result shown. Somehow, this plant showed a poor correlation between phenolic compounds and antioxidant activity using distilled water extract. Some authors claimed that phenolics are the main contributors to antioxidant activity, but some studies proved that the carotenoid compound affects antioxidant activity (Monteiro *et al*., 2020). Hence, other phenolic compounds such as phenolic acids, coumarins, carotenoids, tannins, and others contribute to the antioxidant activity of *A*. *reinwardtii*.

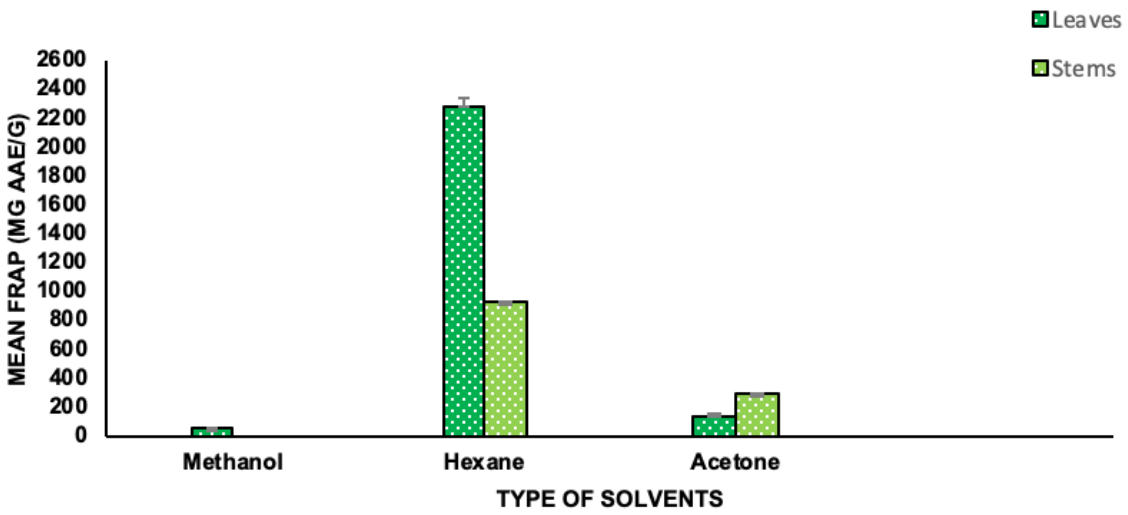

Fig. 8. Effect of extraction method and solvents on Ferric Reduce Antioxidant Power (FRAP) of *Alyxia reinwardtii* leaves and stems crude extracts.

Disc Diffusion Assay (DDA)

The standard antibiotic ciprofloxacin exhibited the highest inhibition zone (mm) in *B. cereus* (11.67 ± 0.23 mm) and *E. coli* (12.00 ± 0.10 mm). As expected, the negative control (DMSO) did not show any activity against any of the bacterial strains. Hexane on leaves extract showed the largest inhibition zone against *E. coli* (11.17 ± 4.48 mm) as shown in Table 3. According to (Asghar *et al*., 2016), ethanolic and methanolic showed the best antibacterial activity against both gram-positive and gram-negative bacteria. This proves its ability in extracting polyphenolic and phytochemicals from natural resources compared to water extract which acts as a domain driver of antibacterial activities since it is hydrophobicity. Several mechanisms cause solvents to show no antibacterial activity that involves modification of cell membrane components, decreasing cell surface hydrophobicity which reduces solvent permeability. Gram-negative minimize the accumulation of solvent molecules inside of the membrane by discarding them from the lipid bilayer using active efflux pumps (Breijyeh *et al*., 2020). This could be the reason why there is no inhibition zone shown by hexane, methanol, and distilled water against *B. cereus*. According to According to Dyrda *et al*. (2019), methanol is toxic to bacterial cells compared to acetone as the membrane of cells cultured in the presence of alcohol is more rigid. The tolerance of bacteria against organic solvents can be also due to stress caused by solvents and also when the microbes have been fit with enzymes that do not lose their activity in the presence of organic media. *Bacillus cereus* produces a solvent-resistant protease that retains at least 95% of its initial activity especially when bacteria is treated by methanol or DMSO. Chemical interaction between solvent with enzymes causes structural deformation and blocks the enzyme's active site, which suppresses the cell's immunity under stressful conditions (Dyrda *et al*., 2019). Somehow, the high zone of inhibition (mm) shown by the extracts only failed to indicate that it possessed the best antibacterial activity. Hence, it is important to test the extracts on MIC and MBC to get precise results.

Table 1. Correlation analysis between extraction yield, phenolic compounds, and antioxidant activity from leaves and stems of *Alyxia reinwardtii*

Plant Parts	Leaves				Stems			
Variables	TPC	TFC	DPPH	FRAP	TPC	TFC	DPPH	FRAP
TFC	-0.534				0.247			
DPPH	0.557	-0.377			0.712*	-0.164		
FRAP	-0.452	0.940**	-0.397		-0.200	0.810	-0.242	
Yield	0.300	-0.645	0.531	-0.581	0.047	-0.700	0.368	-0.729

*Correlation is significant at the 0.01 level (2-tailed)
**Correlation is significant at the 0.05 level (2-tailed)

Table 2. Correlation analysis between extraction yield, phenolic compounds, and antioxidant activity of *Alyxia reinwardtii* solvent extracts

Solvent	Acetone				Methanol				Hexane				Distil Water			
Variables	TPC	TFC	DPPH	FRAP	TPC	TFC	DPPH	FRAP	TPC	TFC	DPPH	FRAP	TPC	TFC	DPPH	FRAP
TFC		0.793				0.227				-0.624				-0.243		
DPPH	-0.262	-0.485			0.913	0.114			-0.403	0.707			-0.383	0.839		
FRAP	0.007	-0.002	0.855		0.978*	0.054	0.955*		-0.330	0.940	0.691		0.258	-0.826	-0.991*	
Yield	-0.840	-0.352	0.125	0.152	-0.469	0.245	-0.142	-0.429	-0.820	0.098	-0.157	-0.204	-0.358	-0.358	0.971*	-0.971

*Correlation is significant at the 0.01 level (2-tailed)
**Correlation is significant at the 0.05 level (2-tailed)

Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC)

Methanol showed the lowest MIC value for leaves, *E. coli* (0.63 µg/mL). MIC value for standard was 1.57 µg/mL for (*B. cereus*) and 4.17 µg/mL (*E. coli*). Methanol extract showed the lowest MBC value which is 2.50 µg/mL against *E. coli* for leaves. The MBC value for the standard was 3.13 µg/mL for (*B. cereus*) and 6.25 µg/mL (*E. coli*) as shown in Table 3. Few solvents did not show an inhibition zone but there are MBC and MIC values. This could be explained by a few mechanisms and properties of natural products such as pH, volatility, solubility, and diffusion in agar which influence the results of agar diffusion assay without affecting broth microdilution assay. If the active compound of the extract is a combination of a few compounds, it causes one of the compounds to lack antibacterial activity when using diffusion assay. There are a few other reasons for such results like extracts poorly soluble in water, too large to move through agar matrix or interact directly with agar polymer and become stuck in place (Balouiri *et al*., 2016).

Table 3. Disc Diffusion Assay (DDA), Minimum Inhibitory Concentration (MIC), and Minimum Bactericidal Concentration (MBC) activity of *Alyxia reinwardtii* stems and leaves crude extracts

	Stem extracts				Leave extracts			
	A[a]	H[b]	M[c]	DW[d]	A[a]	H[b]	M[c]	DW[d]
DDA	Diameter of Inhibition Zone (mm ± SD)							
E. coli	8.67 ± 1.41	9.67 ± 1.41	9.67 ± 0.94	8.17 ± 0.71	10.17 ± 2.12	11.17 ± 4.48	9.17 ± 1.18	9.59 ± 2.00
B. cereus	7.00±0.00	-	-	-	9.17 ± 0.71	-	-	-
MIC	Concentration (µg/mL)							
E. coli	34.25	1.13	1.75	3.75	58.38	1.75	0.63	3.13
B. cereus	68.50	2.25	NV	NV	NV	NV	1.25	6.25
MBC	Concentration (µg/mL)							
E. coli	233.50	7.00	2.50	6.25	137.00	9.00	14.00	30.00
B. cereus	116.75	3.50	NV	NV	NV	NV	7.00	7.50

[a]Acetone, [b]Hexane, [c]Methanol, [d]Distilled Water

(-): Not Evaluated

(NV): Not Valid

Nevertheless, water extracts usually show higher yields compared to ethanolic extracts of plants due to their high polarity. It helps to rupture plant cell walls to induce the release of compounds and molecules into the solvent. According to Nagwa *et al*. (2019), water and methanol extracts of *Matricaria oleifera* and *Matricaria recutita* showed better activity against sensitive and resistant isolates compared to aqueous extract. MIC value should be equal to MBC (bactericidal) or lower than MBC value (bacteriostatic). This is because more amount (concentration) is needed to completely kill a bacterium than to just inhibit its growth. Hence, a MIC value greater than the MBC value is considered as not valid in this study. From the result shown, the MBC value is higher than the MIC value because the extracts could only act as bacteriostatic agents rather than bactericidal. At the concentration used, the bioactive compound was unable to kill bacteria or sustain the activity. As reported by Muskhazli *et al*. (2009), this happens because the bioactive compound is not adequate to cause significant mortality or due to the sensitivity of bioactive compound towards certain types of solvent.

Correlation between plant parts and solvents for Disc Diffusion Assay (DDA)

The correlation study analysis between plant parts and solvents of *Alyxia reinwardtii* for Disc Diffusion Assay (DDA) against *Eschericia coli* is presented in Table 4. The correlation studies suggest that A[a]-M[c], H[b]-M[c], H[b]-DW[d] for leaves, and A[a]-DW[d] for stems are highly correlated. This indicates the solvents used exhibited pronounced activity towards the tested microorganism. Meanwhile, the other solvents showed an appreciable positive correlation though not high. Besides, a strong correlation coefficient was also found between plant parts which is 0.542[*] and 0.866[**] against *E. coli* and *B. cereus* respectively. The results could be attributed to the flavonoid compound found in the leaves and stems of *A. reinwardtii* as proven in this study by the DPPH and FRAP assay (Sumayya & Gopinathan, 2022). Flavonoids function as growth inhibitory activity of the plant antibacterial. As shown in Figure 2, stems of *A. reinwardtii* contains p-hydroxybenzoic acid which is a type of phenolic acid. This phenolic acid are recognized due to their antibacterial activities as they contain -OH and methoxy (-OCH_3) structure to diffuse through the bacterial membrane (Alibi *et al*., 2021). Besides, coumarins and its derivatives such as 3-hydrocycoumarin glycoside and scopoletin found in stems of *A. reinwardtii* shown in Figure 2 also found to be a potential pharmacophore-based drug against bacterial disease (Sahoo *et al*., 2021). Hence, the various polyphenolic constituents found in leaves and stems of *A. reinwardtii* were well extracted by the solvents used against the tested bacteria. According to Table 4, all the solvents extracts from leaves showed a higher correlation compared to stems. This shows that leaves of *A. reinwardtii* contain a considerable quantity of phenolic compounds that were found to be the major contributor to their antibacterial activity.

CONCLUSIONS

Results suggested that *A. reinwardtii* is a potential source of antioxidant and antibacterial agents. Methanolic and hexane were good solvents as they showed the highest crude yield for both antioxidant and antibacterial activity. However, further studies are needed to identify the active ingredients for

profiling to substantiate the true potential of this species as a candidate source of quality antioxidant or antibacterial candidate drugs.

Table 4. Correlation analysis between plant parts and solvents of *Alyxia reinwardtii* for Disc Diffusion Assay (DDA) against *Eschericia coli*

Plant parts	Solvents		Correlation coefficient
Leaves	A[a]	H[b]	0.89
		M[c]	0.999
		DW[d]	0.693
	H[b]	A[a]	0.89
		M[c]	0.932
		DW[d]	0.978
	M[c]	A[a]	0.999
		H[b]	0.932
		DW[d]	0.759
	DW[d]	A[a]	0.693
		H[b]	0.978
		M[c]	0.759
Stems	A[a]	H[b]	0.749
		M[c]	0.343
		DW[d]	0.988
	H[b]	A[a]	0.749
		M[c]	0.879
		DW[d]	0.561
	M[c]	A[a]	0.343
		H[b]	0.879
		DW[d]	0.217
	DW[d]	A[a]	0.988
		H[b]	0.561
		M[c]	0.217

[a]Acetone, [b]Hexane, [c]Methanol, [d]Distilled Water

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CONFLICTS OF INTEREST
The authors declared no conflict of interest.

ETHICAL STATEMENT
Not applicable.

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