Effect of different solvent extracts on the yield and *in vitro* antibacterial activity, with GC-MS analysis of active extracts from *Vernonia amygdalina* leaves

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Abstract:

Introduction: *Vernonia amygdalina* (*V.amygdalina*), commonly known as 'Bitter leaf', is a small perennial shrub with various medicinal properties including the treatment of stomach disorders, fever symptoms, diabetes, hypertension, and coughs. *V.amygdalina* is a potent source of antibacterial properties that may be beneficial in preventing bacterial infections and associated diseases such as fever and diarrhoea. *V. amygdalina* has been extensively studied for its antioxidant, anti-inflammatory, antifungal, anticancer and antidiabetic properties. Although existing literature emphasises the importance of selecting appropriate extraction solvents to preserve the quality and therapeutic properties of *V. amygdalina*, the ideal solvent for maximum extraction of bioactive components remains unclear. **Aim:** The present study aims to determine the most efficient solvent for producing antibacterial-rich *V. amygdalina* extracts. **Methodology:** Dried leaves of *V. amygdalina* were extracted using various solvents (methanol, ethanol, and dichloromethane) to assess the extraction yield. The antibacterial potential of all extracts against *Staphylococcus aureus*, *Escherichia coli* and *Pseudomonas aeruginosa* was evaluated using the agar disc diffusion assay. The profiling of the active extract was accomplished using gas chromatography coupled with mass spectrometry (GC-MS). **Results:** Among the solvents tested, ethanol exhibited the highest extraction productivity, with a percentage yield of 49.20%. However, the findings of this study revealed that dichloromethane is the most efficient solvent for extracting antibacterial compounds from *V. amygdalina* leaves, with the inhibition zones against all bacteria strains ranging from 16.67 ± 1.20 mm to 21.33 ± 0.5 mm at 200 mg/mL. The GC-MS analysis of the dichloromethane extract identified compounds such as phytol, flavonoids, vitamin E, and squalene. **Conclusion:** The choice of the extraction solvent greatly affects the antibacterial efficacy of *V. amygdalina* leaves. The efficacy of *V. amygdalina* against pathogenic bacteria strains demonstrated in this study highlights its potential for further exploration in pharmaceutical applications.

Keywords: Bitter leaf, extraction solvent, extraction yield, zone of inhibition, agar disc diffusion

Introduction:

Bacterial infections represent a significant burden on public health, affecting individuals of all ages and geographical regions (Baker, et al., 2021). Bacterial pathogens may cause a wide range of diseases, from common ailments such as urinary tract infections, respiratory tract infections, and skin infections to more severe conditions like sepsis and pneumonia. Bacterial infections can also complicate the management of chronic conditions, surgical operations, and immunosuppressive therapies, providing additional challenges in patient care. In order to overcome the problem, the global annual production of antibiotics is estimated to reach 100-200 thousand tonnes, with a total production of more than one billion tonnes since 1940 (Serwecińska, 2020). The extensive use of antibiotics has led to a significant rise in the excretion and environmental release of these drugs, thereby fueling the development of drug resistance in bacterial strains. Many antibacterial compounds, either synthetic or natural, have been developed to treat and combat infectious pathogens. However, the emergence of multidrug-resistant bacteria has further impacted the availability and affordability of many of the antibiotics currently prescribed worldwide. Consequently, the treatment will become less effective and increase morbidity, mortality, and medical costs. Therefore, finding a new antibiotic derived from nature is important to combat the harmful effects of multidrug-resistant pathogens.

Vernonia amygdalina (V. amygdalina), commonly known as "bitter leaf", is a perennial shrub of the Asteraceae family with dark green leaves and rough bark. It is native to tropical Africa although it has been cultivated in many parts across West Africa. The plant may reach a height of 1-6 meters. *V. amygdalina* is a multifunctional and fast-regenerating softwood shrub containing anti-nutritional compounds that contribute to its bitter taste. Nevertheless, its high mineral and vitamin content make it a valuable part of the human diet (Oyeyemi, et al., 2018). Traditionally, the leaves of *V. amygdalina* have been used to treat a variety of diseases, including hypertension, measles, constipation, uterine mobility induction, post-partum haemorrhage control, fever, viral disease and hypercholesterolemia. Moreover, it is also reported to be used to treat malaria, venereal illnesses, wounds, hepatitis, jaundice, and diabetes (Nursuhaili, et al., 2019). Previous pharmacological studies have reported *V. amygdalina* exhibiting antioxidant, antifungal, anti-inflammatory, anticancer, antidiabetic, hepatoprotective, neuroprotective, antimalarial, and antibacterial activities (Ugbogu, et al., 2021). The phytochemical compounds contained in *V. amygdalina* include alkaloids, saponins, terpenes, lignans, flavonoids, phenolic acids, steroids, anthraquinone, coumarins, sesquiterpenes, xanthones and edotides. However, the concentration and stability of these bioactive compounds can be influenced by pre- and post-harvest factors, such as drying processes and extraction solvents (Ejike and Ndukwu, 2017).

The extraction of natural bioactive compounds from plant materials is a crucial step in the isolation and separation processes. The most popular approach is solvent extraction and it is critical to select solvents that can effectively extract the relevant phytochemical constituents. Several factors, such as solvent properties, solvent-to-solid ratio, particle size of the plant material, extraction duration and extraction temperature should be considered as they may affect the extraction efficiency (Zhang, et al., 2018). Therefore, the purpose of this study is to extract bioactive compounds from *V. amygdalina* leaves using three different solvents, namely dichloromethane, methanol, and ethanol by using Soxhlet extraction. In addition, the study aims to determine the most potent antibacterial extracts followed by the determination of the bioactive compounds in the active extract of *V. amygdalina* using GC-MS.

Materials and Methods:

Sample preparation and extraction

Fresh *V. amygdalina* leaves were collected from the plants by the hand-picking method. Only healthy, disease-free leaves were selected and thoroughly washed under running tap water. The leaves were then dried in an oven for seven days before being pulverized into a fine powder using a blender and weighed. The powder samples were then stored in a Schott bottle and kept refrigerated until further use.
For the experiment, 35 g of the powder samples were placed in three thimbles. Each thimble was stuffed with cotton wool to prevent the sample from escaping before being placed in a Soxhlet extractor. Three solvents (dichloromethane, ethanol, and methanol) were used, with 300 mL of each being added to three round bottom flasks connected to the Soxhlet extractor and condenser. The Soxhlet extraction was carried out for nine hours. After the extraction was completed, the solution containing solvents and extracts was concentrated using a rotary evaporator to separate the solvent from the crude extracts. The extraction process was repeated three times. Finally, the extraction yield was determined using the following equation:

$$Y = \frac{\text{Weight of dried extract (g)}}{Dry\ weight\ of\ plant\ material\ (g)} \text{ x } 100\%$$

Preparation of bacteria inoculum

One gram-positive (*Staphylococcus aureus*) and two gram-negative (*Escherichia coli* and *Pseudomonas aeruginosa*) bacterial strains were used in this study. A single colony of each bacteria strain was cultivated on nutrient agar followed by incubation at 37°C for 24 hours. Then, one colony of each bacteria strain from the fresh bacterial subculture was transferred into 40 mL nutrient broth solution and incubated at 37 °C for 24 hours. After 24 hours, the broth containing the bacteria was stored in the chiller until further use.

Agar disc diffusion assay

The agar disc diffusion assay was carried out according to Balouiri, et al. (2016) to determine the antibacterial activity of *V. amygdalina* leaves extract. Various dilutions were achieved by reconstituting the *V. amygdalina* extracts in methanol using a two-fold dilution method to achieve concentrations of 25, 50, 100, and 200 mg/mL. 20 µL of each extract concentration was impregnated on the filter paper disc (6 mm in diameter) and left to dry. On nutrient agar plates, 200 µL of each bacterial strain were inoculated evenly using a sterile hockey stick spreader. The filter paper discs containing extracts were then placed on the bacteria-containing agar surface and incubated for 24 hours at 37 °C. The procedure was repeated three times for each bacterial strain. After 24 hours, the diameter of the inhibition zones on each plate was measured. Filter paper discs containing methanol were used as negative controls, whereas antibiotic discs containing gentamycin served as positive controls.

Gas Chromatography-Mass Spectrometry analysis

The dichloromethane extract was analysed using Agilent GC-MS equipment (Agilent 5975C inert, USA) together with a mass spectrometer MS 597C according to the method by Kalaiyarasan and Hariram (2021) with minor modification. The GC-MS is equipped with a DB-1MS capillary column length of 30 m, an inner diameter of 0.25 mm, and a film thickness of 0.25 µm. The initial temperature was 35°C and was raised to 95°C before reaching the final temperature of 300°C. Helium was used as the carrier gas with a flow rate of 1.0 mL/min and an ionisation voltage of 70 eV. The sample injection volume was 1 µL of extract solution, and the analysis was performed for 30 minutes. The chemical components in the extracts were identified by comparing the obtained spectra with those available in the NIST 05a library database (National Institute of Standards and Technology).

Statistical analysis

Data were analysed using Statistical Analysis System (SAS) software for Windows. Differences in means were determined using one-way ANOVA. Results were expressed as a mean of three determinations ± SD. The significance level was set as $p < 0.05$. Tukey's HSD method was used for pairwise mean comparisons.

Results:

Effects of different solvents on extraction yield

Table 1 shows the effect of the extraction solvents on the extract yield of *V. amygdalina* leaves. The highest extraction yield was observed for ethanol extracts at 49.20%, followed by methanol extracts at 45.89%, and dichloromethane extracts at 7.69%.

Table 1. Extraction yield of *V. amygdalina* extracts.

Solvents	Dry weight of plant material (g)	Weight of dry extract (g)	Extraction yield (%)
Dichloromethane	35	2.69 ±0.16	7.69±0.45
Ethanol	35	17.22 ±0.33	49.20±0.93
Methanol	35	16.06±1.03	45.89±0.74

Values are expressed as mean ± SD (n=3).

Disc diffusion assay

Figure 1 presents the results of the disc diffusion assay of ethanol, methanol, and dichloromethane extract of *V. amygdalina* leaves against each bacterial strains used. Results showed that all extracts inhibited the bacterial strains at different concentrations ranging from 25 - 200 mg/mL.

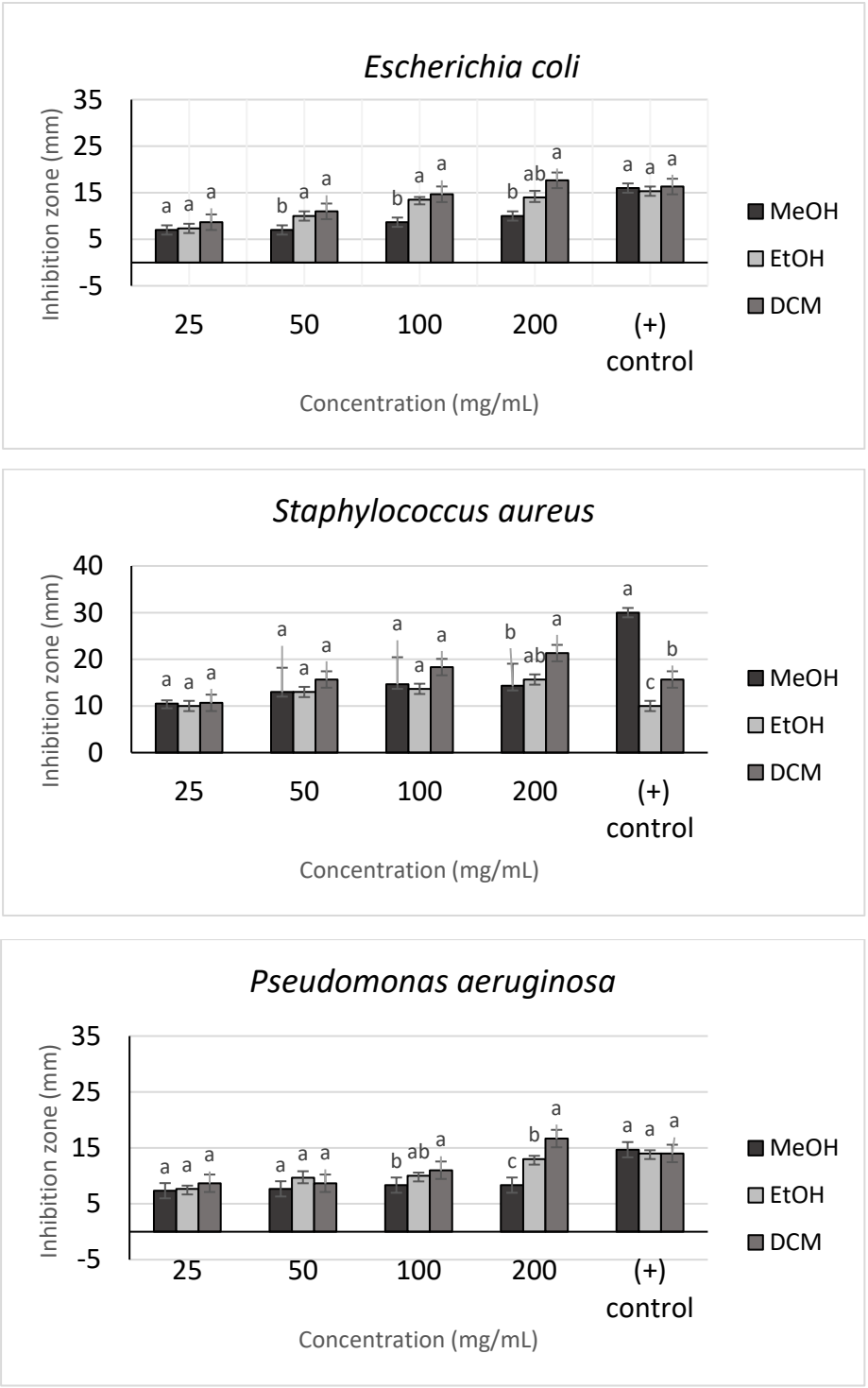

Figure 1. The zones of inhibition of three different solvents at different concentrations against selected bacteria. (+) control: positive control (gentamycin) at 10 ug/mL. Mean values with the same lowercase letter are not significantly different ($p > 0.05$) according to Tukey's HSD Test.

GC-MS Analysis

The GC/MS analysis was performed on the most active extracts of *V. amygdalina*, and the identified chemical constituents were listed with their corresponding retention time and peak area. Specifically, the GC/MS analysis of *V. amygdalina* indicated that the dichloromethane extract contained 26 compounds, as illustrated in Figure 2. Meanwhile, the ten most abundant compounds in the dichloromethane extract, as detected by GC-MS analysis are tabulated in Table 2.

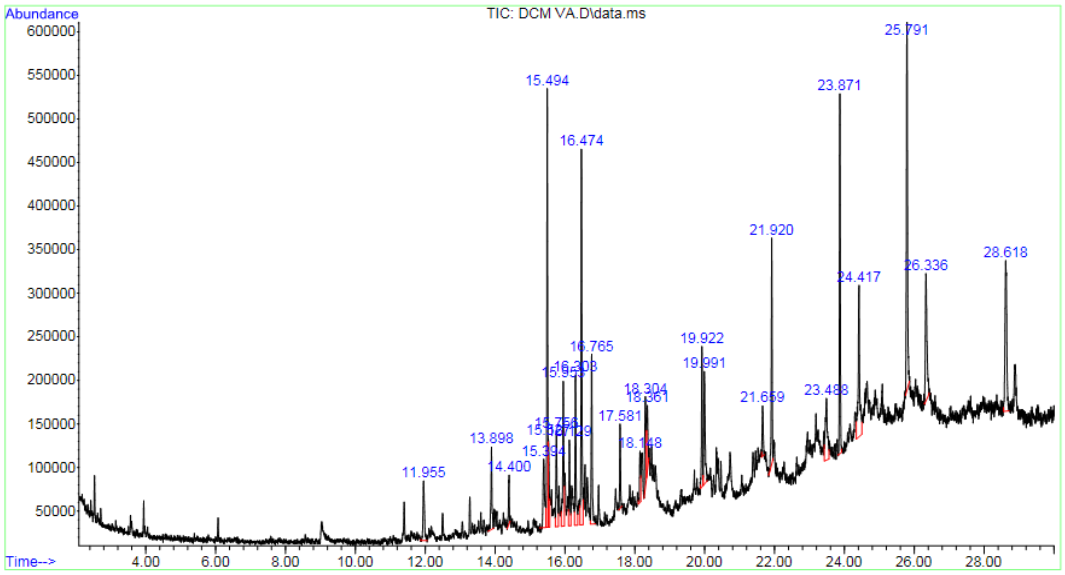

Figure 2. GC-MS chromatogram of the dichloromethane extract of *Vernonia amygdalina* leaves.

Table 2: Chemical constituents present in the dichloromethane extract of *Vernonia amygdalina* leaves.

Retention time (min)	Compound Name	Peak area (%)
25.791	Octadecane, 1-iodo-	9.8189
15.496	Bicyclo[3.1.1]heptane, 2,6,6-trimethyl-, (1.alpha.,2.beta.,5.alpha.)-	8.709
16.476	7,9-Di-tert-butyl-1-oxaspiro (4,5) deca-6,9-diene-2,8-dione	8.434
23.872	2,6,10,15,19,23–hexamethyl-2,6,10,14,18,22-tetracosahexaene (Squalene)	7.336
28.616	Methylenebis(2,4,6-triisopropylphenylphosphine)	6.326
24.417	Eicosane	5.615
26.336	Vitamin E	4.748
21.922	Phthalic acid, 2-ethylhexyl tridecyl ester	4.709
16.764	.beta.-Methylfentanyl	4.027
15.952	3,7,11,15-Tetramethyl-2-hexadecen-1-ol (Phytol)	3.882

Discussion:

The extraction yield revealed that ethanol exhibited the highest percentage yield at 49.20 % followed by methanol (45.89 %) and dichloromethane (7.69 %). This is because the content of bioactive compounds in the extract can vary greatly due to differences in the polarity of the extraction solvent. Higher extraction yields were observed for ethanol and methanol extracts compared to dichloromethane extract, suggesting that the extraction efficiency favours more

polar solvents (Truong, et al., 2019). The presence of hydroxyl groups in ethanol and methanol increases their ability to form hydrogen bonds and other polar interactions, thereby increasing their ability to dissolve a broader range of polar and semi-polar compounds (Srikacha and Ratananikom, 2020). In contrast, the inability of dichloromethane to form hydrogen bonds restricts its interactions with polar functional groups. This finding is consistent with a previous study by Ekam, et al. (2010), which reported a positive correlation between solvent polarity and extraction yield. Additionally, the lower extraction yield may result from the thermal decomposition of phytochemical compounds during the Soxhlet extraction process, as reported by Danlami, et al. (2014). Thermally sensitive phytochemicals compounds undergo degradation during the heating, resulting in low extraction yields (Danlami, et al., 2014).

Figure 1 indicates that *S. aureus* was the most susceptible to all solvent extracts at all concentrations, with an inhibition zone diameter ranging between 7 to 22 mm. A previous study by Bukar, et al. (2013) reported that the most susceptible bacteria strains to the ethanolic extract of *V. amygdalina* are *S. aureus* followed by *E. coli* and *P. aeruginosa*. The variation in inhibition zones can be attributed to the differences in the structure of the cell envelope between Gram-positive and Gram-negative bacteria (Fivenson, et al., 2023). The higher susceptibility observed in the *V. amygdalina* extracts may be associated with the presence of two cellular membranes, with an outer membrane coated with lipopolysaccharides in Gram-negative bacteria, acting as a powerful barrier that restricts the penetration of compounds. In contrast to the barrier of Gram-negative bacteria, the absence of an outer membrane in Gram-positive bacteria reduces the interference and facilitates the diffusion of compounds across the cell membrane (Fivenson, et al., 2023).

Among various solvents tested, dichloromethane extract displayed the highest antibacterial activity against all bacterial strains at various concentrations (25-200 mg/mL) when tested by the agar disc diffusion assay (Figure 1). However, the antibacterial activity of dichloromethane extract is not significantly different compared to all tested solvents at low concentrations. On the other hand, there are some significant differences at 200 mg/mL. Dichloromethane extracts exhibited inhibitory activity against *E. coli*, *P. aeruginosa* and *S. aureus,* with an inhibition zone ranging from 8 to 22 mm, exceeding the inhibitory activity of methanol and ethanol extracts. The non-polar nature of dichloromethane allows selective extraction of lipophilic or non-polar antibacterial compounds from *V. amygdalina* (Benramdane, et al., 2022). Furthermore, Gram-positive bacteria are more sensitive to lipophilic extracts than Gram-negative bacteria, which explains the larger inhibition zones observed for *S. aureus* in the dichloromethane extract (Benramdane, et al., 2022). Further GC-MS analysis of the dichloromethane extract revealed a predominance of lipophilic compounds as shown in Table 1. The identified compounds included those known for their antibacterial properties, such as squalene, phytol, β-methylfentanyl and octadecane (Hartmann, et al., 2020; Huang, et al., 2021; Olusola, et al., 2021; Oladunmoye, et al., 2019; Unlu, et al., 2021; Wijayanti and Dewi, 2022). These compounds may have a greater impact on the inhibition of bacterial growth in the dichloromethane extract.

Conclusion:

Our findings highlight the influence of solvent selection on the extraction yield and antibacterial activity of *V. amygdalina* leaves extract. Ethanol and methanol, being more polar solvents, exhibited higher extraction yields, while dichloromethane selectively extracted lipophilic compounds due to its non-polar nature. Among the solvents tested, dichloromethane was found to be the most effective solvent for the production of antibacterial-rich extract, exhibiting potent antibacterial activity against *Staphylococcus aureus*, *Escherichia coli*, and *Pseudomonas aeruginosa*. In addition, GC-MS analysis identified 26 phytochemical compounds with potential antibacterial properties. Given the increasing demand for plant-derived antibacterial agents as a substitute for synthetic antibiotics, *V. amygdalina* may be a promising source for the development of new antibacterial drugs to prevent a variety of diseases. Future research may focus on the characterization of the active compounds as well as the elucidation of their mechanism of action for the development of antibiotics from *V. amygdalina*.

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