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Extraction, Isolation, Phytochemical Profiling, and Biological Evaluation of Root Extracts of *Aquilaria malaccensis*

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ABSTRACT

Aquilaria malaccensis is a medicinally valuable plant traditionally used in various systems of medicine to manage a wide range of ailments. This study focuses on the extraction, isolation, and phytochemical screening of ethanol and methanol root extracts of *A. malaccensis* to investigate their potential biological activities and identify bioactive phytoconstituents. Preliminary phytochemical screening was carried out to identify the presence of major classes of bioactive constituents is flavonoids, alkaloids, glycosides, and steroids. Isolation of phytoconstituents was performed using suitable chromatographic techniques, and the isolated compounds were characterized by IR, ^{1}H NMR, and Mass analytical methods. The phytochemical analysis revealed that flavonoids are the main constituent in both the extracts, with variations in their abundance depending on the solvent used. Based on the phytochemical composition, the ethanol and methanol root extracts and isolated compounds like **MAME-02 and EAME-01** were evaluated for their possible biological activities, including antibacterial, antioxidant, and anthelmintic using established in vitro methods. The results indicated that both extracts exhibited significant biological activities, with the ethanol extract showing comparatively higher efficacy in antibacterial and anthelmintic activities.

1. INTRODUCTION

Medicinal plants represent an important and diverse source of bioactive compounds with extensive applications in pharmaceutical, cosmetic, and nutritional fields. Their therapeutic use dates back thousands of years, with historical evidence indicating their utilization as natural remedies as early as 4000–5000 B.C. Ancient Chinese civilization was among the earliest to systematically document and employ medicinal herbs for the management of various ailments. Likewise, the traditional medical systems of India provide extensive

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evidence of the therapeutic application of plants[1]. Ancient Indian texts, including the *Rig Veda*, dating approximately to 1600–3500 B.C., contain references to the medicinal use of numerous plants, underscoring the longstanding role of medicinal plants in traditional healthcare and their continued importance as valuable sources for therapeutic discovery [2-3]. Medicinal plants represent an important and enduring component of healthcare systems worldwide, owing to their extensive use in the prevention and treatment of a wide range of human diseases. Ethnobotanical investigations serve as an important source of knowledge for the identification of medicinally valuable plants and the discovery of bioactive compounds with potential therapeutic applications. Natural products obtained from diverse biological sources have made substantial contributions to both traditional and modern medicine and continue to provide valuable leads for drug discovery and development. Herbal medicines are derived from a variety of natural sources, predominantly medicinal plants, as well as other biological resources such as microorganisms [4-7].



Fig 1. Plant of *Aquilaria malaccensis*

Aquilaria spp. are the most valuable and highly fragrant forest products locally known as agarwood, aloes wood, eaglewood, guacharo, kalamabak or oudh depending on the region [8]. Several genera that might be a source of agarwood production from the family Thymelaeaceae might be endangered due to the deterioration of their natural resources include *Aquilaria, Gonystulus*, and *Gyrinops,* having been listed in Appendix II of the Convention on Internal Trade in Endangered Species of Wild Fauna and Flora [4]. In Malaysia and Indonesia. Due to a limited resource base and high demand, *Aquilaria malaccensis,* the primary source of agarwood in India. "{The export of agarwood requires an Export Permit from designated CITES Authorities. Such a permit can be issued on the basis of a Certificate of Legal Procurement (CLP) issued by an officer not below the rank of Assistant Conservator of Forests. The generic name *Aquilaria* originates from the Latin word 'aquila,' meaning 'eagle,' or from the Akkadian word 'eklu,' meaning 'dark' or 'ekelu,' meaning 'to be dark'. The specific epithet "*malaccensis*" refers to its probable type locality, Malacca, a coastal state of Malaysia [7]. It is highly sought after for its resin and essential oils, while less has been focused on the health-beneficial effects of other parts of the plant despite the various ethnopharmacological evidence. Previous scientific research on A. malaccensis reported its potential pharmacological activities [6], including anticancer, antimicrobial, antioxidant, anti-inflammatory, immunomodulatory, antidiabetic, embryogenesis, and antitrypanosomal activity.

2. MATERIALS AND METHODS

All chemicals and reagents used in the study, including ethanol, methanol, distilled water, extraction and chromatographic solvents, 2,2-diphenyl-1-picrylhydrazyl (DPPH), Whatman filter paper, silica gel, and ascorbic acid, were of analytical grade and used as received. Nutrient agar and other microbiological media were obtained from certified suppliers, including Invitrogen, Sigma-Aldrich, and HiMedia Laboratories Pvt.

Ltd., Mumbai, India. The principal equipment employed in the study comprised a mechanical grinder, Soxhlet apparatus, maceration setup, analytical balance, rotary evaporator, and hot-air oven.

Plant Collection and Extraction: Fresh roots of *Aquilaria malaccensis* were collected and authenticated by a qualified botanist. The roots were washed, shade-dried, powdered, and stored in airtight containers. The powdered material was separately extracted with ethanol and methanol using a Soxhlet apparatus and maceration setup. The extracts were filtered, concentrated under reduced pressure, dried, weighed, and stored at 4°C until further analysis. The percentage yield was calculated based on the weight of the dried extract relative to the initial plant material.

Phytochemical Screening

The ethanol and methanol root extracts of whole plant powder were carried out using standard laboratory procedures to detect the presence of different secondary metabolites like alkaloids, flavonoids, phenolics, tannins, saponins, glycosides, terpenoids, steroids, carbohydrates, proteins, and reducing sugars.

Isolation of Plant extract: The root extracts of *Aquilaria malaccensis* powder were powdered, weighed (200g) and filled in solvent extraction Soxhlet tools. Using petroleum ether, the powdered substance was defatted. The defatted powder was dried at room temperature and extracted using ethanol and methanol. Solvents were evaporated to get the dried residue of the extracts. A small portion of both crude extracts was dissolved in chloroform, and the solution is spotted on TLC plates. Then these plates are run by specific solvents system and viewed individually under UV light and also with the 10% H_2SO_4 reagent. A portion of the extracts (2g) is subjected to column chromatography (silica gel, 2x90 cm). Finally, the column is eluted with the starting 2:8 to 4:6 solvent system of n-hexane and ethyl acetate to give one major compound(250mg) and the remaining two compounds in small quantities (2mg and 3mg). Identification and analytical, standard spectroscopic methods for investigating the structure of the isolated compounds (MAME-02 and EAME-01) were used, including FT-IR, ^{1}H NMR, ^{13}C NMR, and mass spectrometry.

Antibacterial Activity: Agar diffusion method (Cup plate method)- The antibacterial activity was screened by using diffusion method. The standard cultures of gram-positive *Staphylococcus aureus*, *Bacillus subtilis* (Gram positive) and *Escherichia coli*, *Klebsiella pneumonia* (Gram negative) species were obtained from Department of Microbiology, Kakatiya University, Warangal. In this method the Petri dishes were filled with inoculated liquefied agar medium to uniform thickness the pits or bores were made using core borer which filled with test compounds and a Standard drug and inoculated at 37 ± 1ºC hrs. The drug will diffuse into the agar medium are prevents the growth of microbes and produce a clear zone of inhibition. The stock solutions of newly synthesized compounds and standard Ampicillin were prepared in dimethyl sulfoxide at a concentration of 100µg/0.1 ml.

Antioxidant Activity: Antioxidant activity was determined using the DPPH radical-scavenging assay. Different concentrations of the extracts were incubated with DPPH solution, and absorbance was measured at 517 nm. Ascorbic acid was used as the standard. The percentage inhibition and IC_{50} values were calculated.

The antioxidant activity of the extracts was evaluated using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay. Briefly, a DPPH solution (100 µg/mL; 0.2 mmol/L) was prepared in methanol and mixed with the methanolic solutions of the extracts or standard compounds at different concentrations (5, 10, 30, 40, 50, and 100 µg/mL) [15–18]. The reaction mixtures were thoroughly mixed and incubated at room temperature for 30 min in the dark. The absorbance of the remaining DPPH radicals was measured at 517 nm using an appropriate spectrophotometer. Ascorbic acid (vitamin C) was used as the reference standard. The percentage of DPPH radical scavenging activity was calculated using the following equation:

$$\textbf{Scavenging percentage of each extract} = (AB–AA)AB)\times 100$$

where AB is the absorption of blank sample, AA is the absorption of test sample

Higher percentage inhibition indicates greater DPPH radical scavenging activity and, consequently, stronger antioxidant potential.

Anthelmintic Activity: The Isolated biomolecules are screened for anthelmintic activity by using earthworms (*Phreesia posthuman)*. One earthworm is placed in the standard drug solution and test compound's solutions at room temperature. Normal saline used as control. The standard drug and test compounds were dissolved in minimum quantity of dimethyl sulfoxide (DMSO) and adjusted the volume up to 10 ml with normal saline solution to get the concentration of 0.1% w/v, 0.2 % w/v and 0.5% w/v. Albendazole was used as a standard drug. The Isolated biomolecules were evaluated by the time taken for complete paralysis and death of earthworms.

Statistical Analysis

All experiments were conducted in triplicate, and the results were expressed as mean ± SD. Statistical significance was evaluated using one-way ANOVA followed by an appropriate post-hoc test, with $p < 0.05$ considered statistically significant.

3. RESULTS AND DISCUSSION

3.1. Fluorescence Analysis: The powdered samples of leaf part of plant Urtica dioica L treated with water, 10% NaOH, 50% H2SO4 and 1N HCl. The fluorescence of these extracts were observed under ordinary visible light and also under UV light (254 nm) and recorded.The powdered samples of leaf part of plant *Aquilaria malaccensis* treated with water, 10% NaOH, 50% H_2SO_4 and 1N HCl. The fluorescence of these extracts were observed under ordinary visible light and also under UV light (254 nm) and recorded.

3.2. Preliminary Phytochemical Screening of ethanol and methanol Extract of Roots of *Aquilaria malaccensis*. Standard methods were used for the identification of this preliminary phytochemical screening of the both root extracts of *plant* revealed the presence or absence of secondary metabolites. The qualitative analysis showed the presence of Alkaloids, Glycosides, Phytosterols, and Flavonoids compounds in the ethanol and methanol extracts with varying intensities. As shown results, the majority of these secondary metabolites are present in the both extracts are Flavonoids.

Table 1: Fluorescence Analysis of Root powder of *Aquilaria malaccensis* Plant

S.No	Samples	Treatment with chemical reagents	Under ordinary light	Under UV light-254nm
1	Root powder	Powdered with water	Light white	White
2	Root powder	powder with 1N NaOH solution	Pale green	Green
3	Root powder	Powder with 50% H2SO4 solution	Thick brown	Dark brown
4	Root powder	Powder with 1N HCl solution	Thick Yellow	Light Yellow

Table 2: Preliminary phytochemical Screening**.**

Test	Root of *Aquilaria malaccensis*	
	Methanol Extract	**Ethanol Extract**
Alkaloids	+	+
Glycosides	+	-
Carbohydrates	-	-
Phenolic compounds	-	-
Steroids	-	+
Flavonoids	+++	+++
Terpenes	-	-
Saponins	-	-

Table 3: Physicochemical Parameters

Parameters	**Result of the plant materials (% w/w or v/v)**	**General Standards (Approximately)**
Total Ash	8.32±0.32% w/w	Should not exceed the given std value- **10%w/w**
Acid Insoluble Ash	4.45±1.02% w/w	Should be between 1- 5% w/w.
Water Soluble Ash	2.043±0.42% w/w	Normally, within 5-12%w/w.
Loss on Drying	7.54±1.02% w/w.	Typically, within 5-12% w/w.
Sweeling	42±5.32& v/v	Maximum variable-5-100% v/v

The ethanol extracts showed the highest percentage yield like 15.43% w/w, followed by methanol extracts-7.86% w/w. The Data represent mean values expressed as w/w.

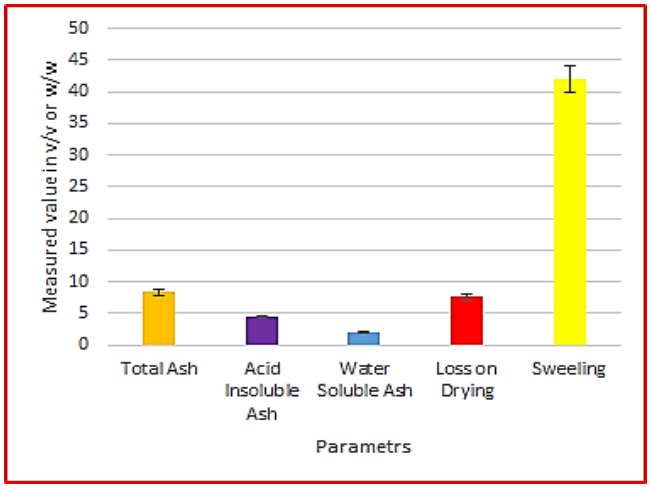


Fig 2. The measured values include various parameters; all are expressed in w/w/ or v/v.

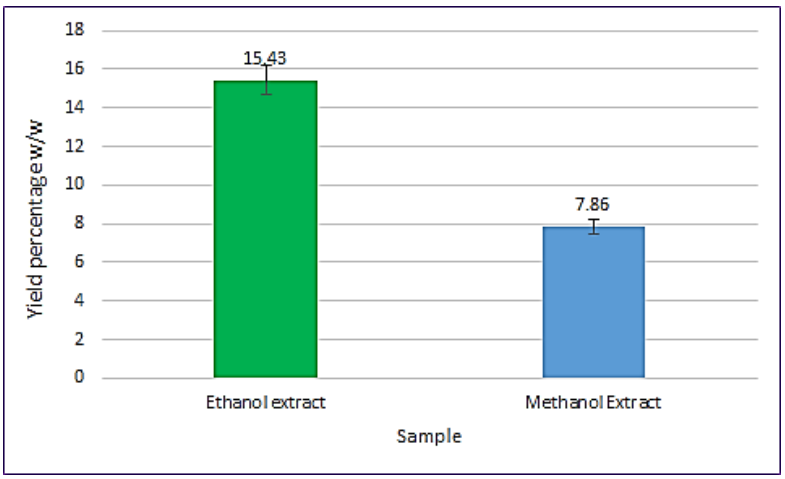
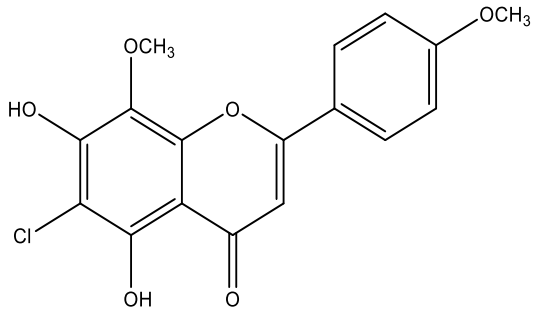


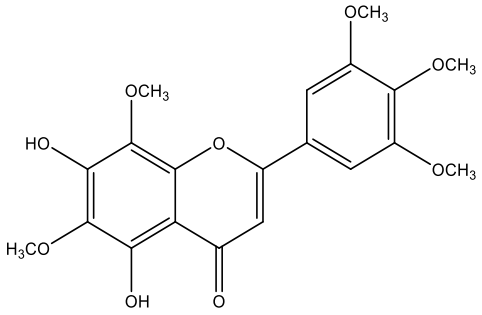
Fig 3. Percentage Yield of ethanol and methanol solvents of *Aquilaria malaccensis.*

3.3. Characterization of Isolated Compounds-Roots of *Aquilaria malaccensis*. Identification and analytical, standard spectroscopic methods for investigating the structure of natural products- ethanol (EAME-01) and methanol (MAME-02) root extracts of *Aquilaria malaccensis* comprise nuclear magnetic resonance (NMR), infrared spectroscopy (IR), and these are often combined with mass spectrometry (MS). In physical and chemical characterization, the EAME-01 isolated as pale-yellow colour powder and MAME-02 was isolated as brown colour powdered. The melting point of both the compounds are 181-183ºC (EAME-01) and 207-209ºC (MAME-02) respectively. **EAME-01:** IR values(cm^{-1}):IR band was observed at 3453(-OH Str, Ar-OH); 3087(-CH Str, Ar-CH); 2976,2899(-CH Str, Alkane); 1727(-C=O Str, Keton group); 1610-C-C Str in Ar-C); 1423(-C=C Str in Ar-C=C), 1078(-C-O Bend). ^{1}H NMR spectrum. It was observed at 12.865(1H, s, Ar-OH); 10.766(1H, s, Ar-OH); 8.253(1H, s, Ar-H); 8.102-8.001(2H, d, Ar-H); 7.869-7.823(2H, d, Ar-H); 3.775-3.683(s, 6H, Ar-OCH_3). **MAME-02:** IR values(cm^{-1}):IR band was observed at 3532, 3484(-OH Str, Ar-OH); 3102(-CH Str, Ar-CH); 2956,2867(-CH Str, Alkane); 1734(-C=O Str, Keton group); 1602(C-C Str in Ar-C); 1419(-C=C Str in Ar-C=C), 1103(-C-O Bend). ^{1}H NMR spectrum. It was observed at 12.643(1H, s, Ar-OH); 10.276(1H, s, Ar-OH); 8.453(1H, s, Ar-H); 8.195-8.102(2H, d, Ar-H); 3.834-3.732(s, 9H, Ar-OCH_3), 3.587-3.521(s, 6H, Ar-OCH_3). The isolated EAME-01 and MAME-02 were characterized by IR, ^{1}HNMR and Mass spectral data**.** The results revealed that the isolated compound was identified as a flavonoid.



6-chloro-5,7-dihydroxy-8-methoxy-2-(4-methoxyphenyl)-4*H*-chromen-4-one
Chemical Formula: $C_{17}H_{13}ClO_6$
Exact Mass: 348.04

Fig 4. Isolated compounds-EAME-01-(Flavonoid related compounds)



Chemical Formula: $C_{20}H_{20}O_9$
Exact Mass: 404.11

5,7-dihydroxy-6,8-dimethoxy-2-(3,4,5-trimethoxyphenyl)-4*H*-chromen-4-one

Fig 5: Isolated compounds -MAME-02 (Flavonoid-related compounds)

3.4. Antioxidant activity:

DPPH Method: The antioxidant activity of the test compounds was evaluated using the DPPH radical-scavenging method. DPPH is a stable purple-colored free radical that is reduced to a colorless form in the presence of antioxidants. Test compounds and ascorbic acid (standard) were prepared in methanol at concentrations of 15, 25, 50, 75, and 100 µg/mL and mixed with DPPH solution. After incubation for 30 min, absorbance was measured at 516 nm. The percentage of radical-scavenging activity was calculated, and the IC_{50} value was determined as the concentration required to inhibit 50% of DPPH radicals.

H_2O_2 Method: Hydrogen peroxide scavenging activity was determined using a spectrophotometric method. Briefly, 1 mL of each test compound (10–100 µg/mL) was added to 0.6 mL of 40 mM hydrogen peroxide prepared in 50 mM phosphate buffer (pH 7.4). After incubation for 10 min, the absorbance was recorded at 230 nm using phosphate buffer without hydrogen peroxide as the blank. Hydrogen peroxide served as the control, while ascorbic acid was used as the reference standard. The percentage of hydrogen peroxide scavenging activity was calculated based on the absorbance values of the control and test samples.

Table 4. Antioxidant Activity- Hydrogen Peroxide method of root extracts and isolated compounds of *Aqilaria Malaccensis* Plant.

Samples	IC50 values
Ethanol extracts	127.47±2.765
Methanol Extracts	185.32±5.854
MAME-02	58.94±3.023
EAME-01	38.51±0.021
Ascorbic acid	24.98±0.12

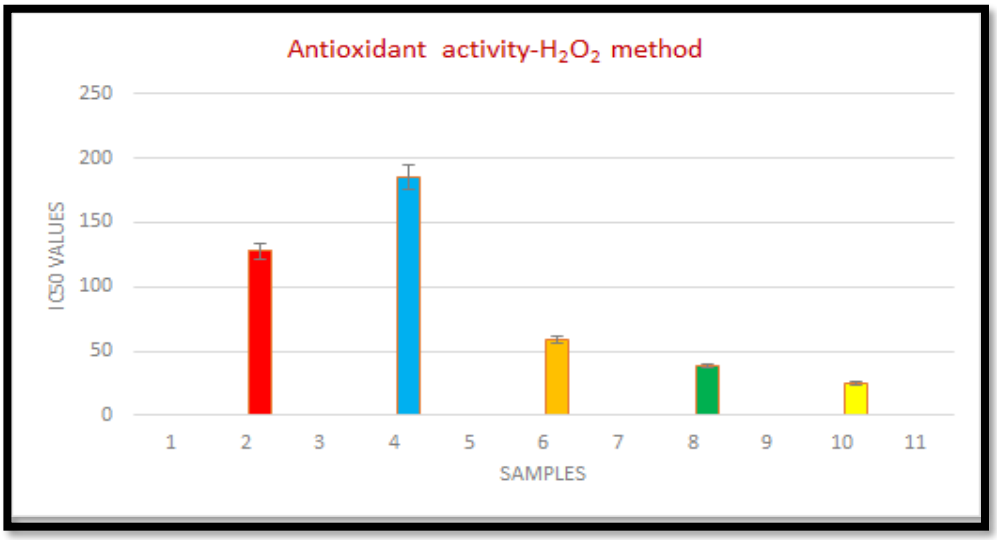


Fig 6. Graphical representation of antioxidant activity- hydrogen peroxide method of root extracts and isolated compounds of *Aqilaria Malaccensis* Plant.

The H_2O_2 scavenging assay showed that the sample exhibited the lowest IC_{50} value, indicating the strongest antioxidant activity, followed by samples EAME-01 and MAME-02. Methanol extract showed the highest IC_{50} value and the weakest activity.

Table 5: Antioxidant Activity- DPPH meyhod of root extracts and isolated compounds of *Aqilaria Malaccensis* Plant.

Samples	IC50 values
Ethanol extracts	102.32±0.653
Methanol Extracts	168.32±3.012
MAME-02	43.82±2.042
EAME-01	26.87±0.021
Ascorbic acid	14.87±0.001

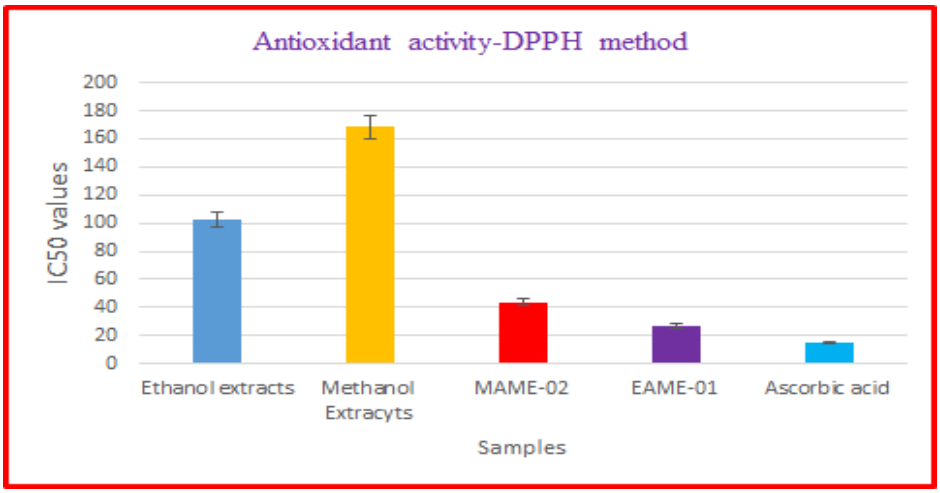


Fig 6. Graphical representation of Antioxidant Activity-DPPH method of root extracts and isolated compounds of *Aqilaria Malaccensis* Plant.

The DPPH assay showed the highest antioxidant activity for ascorbic acid, followed by EAME-01 and MAME-02, based on their lower IC_{50} values. Ethanol extract exhibited moderate activity, whereas methanol extract showed the weakest activity. Lower IC_{50} values indicate stronger free-radical-scavenging activity.

3.5. Anthelmintic activity: The anthelmintic activity of ethanol and methanol extracts and isolated biomolecules was evaluated using earthworms at concentrations of 0.1%, 0.2%, and 0.5% w/v. Albendazole served as the standard drug, while normal saline was used as the control. Paralysis and death times were recorded and compared with the standard drug.

Table 6. Anthelmintic activity-Paralysis time of ethanol, methanol and isolated compounds of *Aqilaria Malaccensis* Plant.

Name of the samples	Time in mints					
	Paralysis time			Death Time		
	0.1%	0.2%	0.5%	0.1%	0.2%	0.5%
Control	0	0	0	0	0	0
Albendazole	16	13	10	43	37	24
Ethanol extracts	20	17	15	55	40	31
Methanol Extracts	32	24	22	67	52	41
MAME-02	22	20	18	49	46	32
EAME-01	16	13	11	44	39	26

The anthelmintic activity results showed that Albendazole and EAME-01 exhibited the strongest activity, with shorter paralysis and death times. Ethanol extracts and MAME-02 showed moderate activity, whereas methanol extracts were less effective. Overall, the activity increased with concentration, and the control showed no activity.

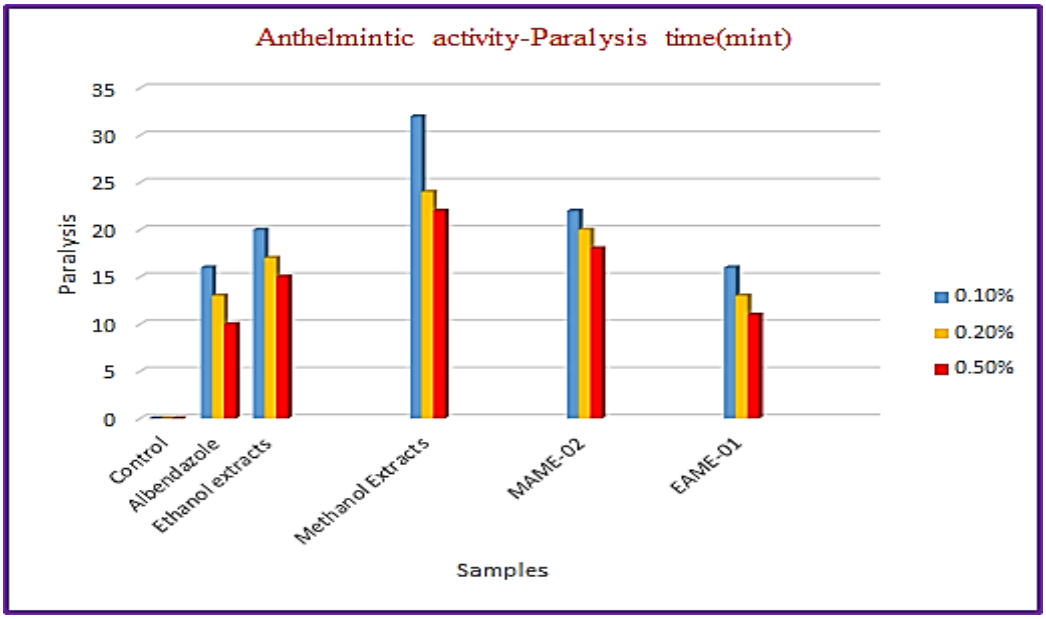


Fig 7: Anthelmintic Activity-Paralysis time of ethanol, methanol and isolated compounds of *Aqilaria Malaccensis* Plant.

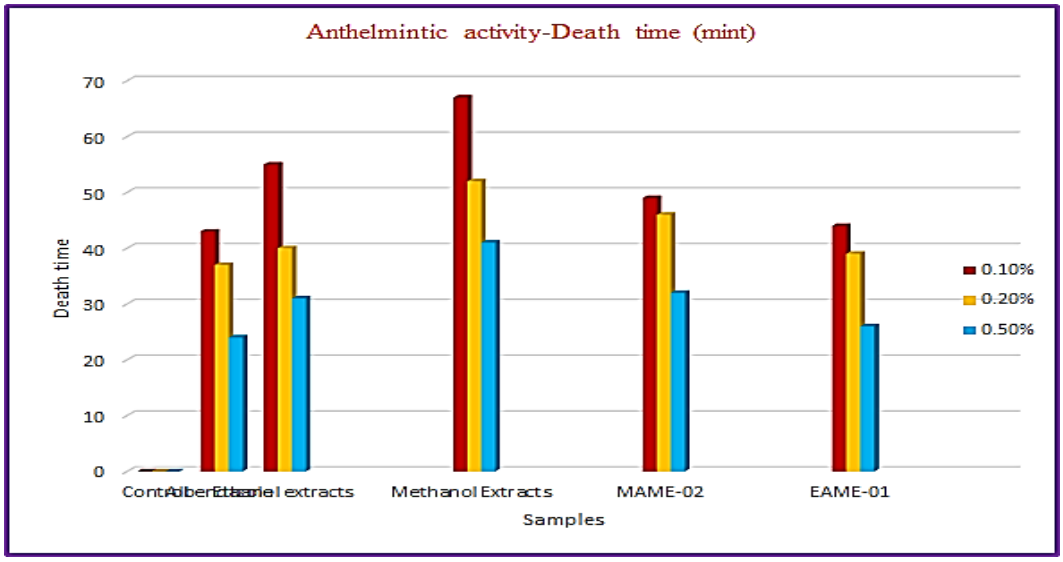


Fig 8. Anthelmintic activity-Death time of ethanol, methanol and isolated compounds of *Aqilaria Malaccensis* Plant.

3.6. Antibacterial activity: Antibacterial activity was evaluated by the agar cup-plate diffusion method against Gram-positive (Staphylococcus aureus, Bacillus subtilis) and Gram-negative (Escherichia coli, Klebsiella pneumoniae) bacteria. Test compounds and standard ampicillin (100 µg/0.1 mL), dissolved in DMSO, were added to agar wells and incubated at 37 ± 1°C. Antibacterial activity was assessed by measuring the zones of inhibition.

Table 7: Antibacterial activity of extracts and isolated-MAME-02 and EAME-01 compounds.

Compound	**Zone of Inhibition (in mm)**			
	Bacillus-subtilis(+Ve)	***Staphylococcus-aureus(+Ve)***	***Escherichia-coli(-Ve)***	***Klebsiella-pneumonia(-Ve)***
Streptomycin	**32**	**28**	**32**	**33**
Methanol extract	13	16	12	20
Ethanol extract	20	22	18	20
MAME-02	19	20	19	22
EAME-01	**25** *	**23** *	**26** *	**28** *

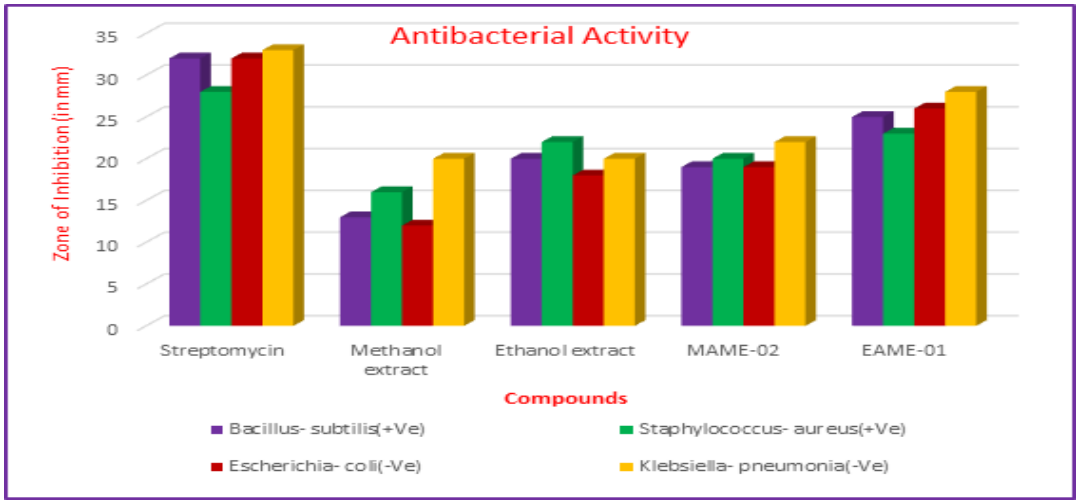


Fig 9. Graphical representation of the Antibacterial activity of plant extracts and isolated products, with their zones of inhibition (mm).

The antibacterial activity graph shows that the standard drug streptomycin exhibited the highest inhibition against all tested bacteria. Among the extracts, EAME-01 showed the strongest overall activity, followed by MAME-02 and the methanol and ethanol extracts, with variations across bacterial strains.

4. CONCLUSION

To the best of our knowledge, the present study provides the first report on the antibacterial and antioxidant activities of purified flavonoid compounds isolated from the ethanolic and methanolic extracts of *Aquilaria malaccensis*. The findings highlight the significant pharmacological potential of *A. malaccensis*, particularly its bioactive flavonoid constituents. The biological activities observed in the extracts may be attributed, at least in part, to the presence of the identified compounds **MAME-02** and **EAME-01**. These findings provide scientific support for the traditional medicinal applications of *A. malaccensis* and underscore the potential of its phytoconstituents as promising sources of bioactive agents. Further investigations are warranted to elucidate their mechanisms of action, establish their safety and efficacy, and explore their potential applications in the development of pharmaceutical and nutraceutical formulations for human and veterinary healthcare.

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