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Original Research Article

ANTIMICROBIAL AND MEMORY ENHANCING ACTIVITIES OF *Byrsocarpus coccineus* SCHUM. AND THONN. AND *Ficus exasperata* VAHL USING BROTH MICRO-DILUTION METHOD

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ABSTRACT

Alzheimer's disease (AD) is a type of dementia with cholinergic neuronal loss, and memory improvement is aided by acetylcholinesterase inhibition. Existing drugs are not curative, leading to interest in alternative treatments. Therefore, this research work assessed the microbial and acetylcholinesterase-inhibiting effects of *Byrsocarpus coccineus* Schum and Thonn as well as *Ficus exasperata* Vahl. Phytochemical screening, brine shrimp lethality assay, modified Ellman's anticholinesterase assay and broth micro-dilution assay of the crude extracts were used to identify the memory-enhancing and antimicrobial properties of the crude extracts, and data were analyzed using GraphPad Prism and One-way ANOVA. *B. coccineus* and *F. exasperata* inhibited AChE at concentration-dependent rates, with *B. coccineus* root showing the highest inhibition at 5.00 mg/mL (97.4 ± 0.8%). *F. exasperata* showed the highest minimum inhibitory concentrations (MIC) against *Staphylococcus aureus, Klebsiella pneumonia. Bacillus subtilis, S. aureus, Escherichia coli, and Klebsiella pneumoniae* all had minimum inhibitory concentrations (MICs) of 12.5 µg/mL, 25 µg/mL, 50 µg/mL, and 50 µg/mL, respectively, while *Candida albicans* and *Trichophyton rubrum* had MICs of 25 µg/mL and 50 µg/mL, respectively. Stem and root of *Byrsocarpus coccineus* and *Ficus exasperata* exhibited acetylcholinesterase inhibiting potentials in a concentration-dependent manner. The leaves, stems, and roots of both plants revealed variations in inhibiting the growth of the tested microorganisms. *Byrsocarpus coccineus* and *Ficus exasperata* may be promising sources and templates for AChE-inhibiting and antimicrobial drug discovery and development.

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INTRODUCTION

Learning occurs through conscious and subconscious interaction with the environment, while memory is the capacity to remember this interaction. Memory encodes, stores, retains, and recalls information, enabling future decision-making based on prior experiences [1]. Acetylcholine, glutamate, GABA, glycine, dopamine, norepinephrine, and serotonin are essential neurotransmitters useful for several bodily processes. However, changes in their levels, synthesis, and homeostasis can lead to neurological diseases like Parkinson's disease, schizophrenia, depression, and Alzheimer's disease. Recent research suggests a central nervous system microbiome.

The gut microbiome, along with microbial metabolites, neurotransmitters, and immunological chemicals, communicates with the brain via pathways like the brain-gut axis. These molecules can affect brain function and behavior

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through various signalling pathways [2]. Changes in the brain's microbiome can impact neurological conditions. Treating comorbid disorders, such as neurodegenerative diseases (NDDs) can involve microbial infection. Studies have investigated the relationship between bacteria and NDDs in Alzheimer's disease (AD) patients [3]. The brain microbiome research is in its early stages, but understanding itsto intricate connections is crucial for understanding the aetiology, development, and potential therapeutic approaches for neurodegenerative illnesses [4].

Plant metabolites have been shown to improve human health by preventing microbial infections and protecting against neurological disorders [3]. As diseases progress and current treatments have adverse effects, research into natural product resources for treating microbial diseases has become necessary [4]. Extracts from medicinal plants have shown acetylcholinesterase inhibitory activity, making them useful for managing neurological illnesses caused by AChE dysregulation [5]. This study aims to assess the potential of *Byrsocarpus coccineus* (FHI 113906) and *Ficus exasperata* a (FHI 113907) for these properties, to record the potential of these plants for both antimicrobial and acetylcholinesterase-inhibiting properties.

Byrsocarpus coccineus Schum. and Thonn. is a climbing shrub commonly known as “crimson thyme” of the Connaraceae family. It is known locally as "Amuje wewe" [6]. In traditional African medicine (TAM), preparations of the plant are used to treat a variety of conditions, including pain, inflammation, wounds, high blood pressure, earache, gonorrhea, impotence, jaundice, piles, diarrhea, and tumors [7].

Ficus exasperata Vahl, is a deciduous tree in the family Moraceae commonly known as “forest sandpaper leaf tree” due to the scaly appearance of its leaf surface. It is locally known as "Eepin". In ethno-medicine, it has been used to treat inflammatory diseases, blemishes, stomachaches, leprosy, ringworm infection and bleeding disorders. The leaf extract has been used to treat wounds, hemorrhage, epilepsy, rheumatism, arthritis, colic, and high blood pressure. Its roots have been used in the management of hemorrhoids, asthma, dyspnea, and venereal illnesses are treated with the roots [8].

METHODS

Microorganisms.

Bacterial pathogens: Gram-negative (*Klebsiella pneumoniae* ATCC 700303 and *Escherichia coli* ATCC 25922) and Gram-positive (*Staphylococcus aureus* ATCC 6571 and *Bacillus subtilis* ATCC 3366) bacteria were acquired from the University of Ibadan's Department of Pharmaceutical Microbiology.

Trichophyton rubrum and *Candida albicans* are fungi acquired from the University of Ibadan's Department of Pharmaceutical Microbiology.

Chemical and Reagents (Sigma-Aldrich, Germany)

Distilled water, Methanol, Phosphate buffer, Acetylcholinesterase enzyme (AChE), Acetylthiocholineiodide (ATCI), Eserine, Ciprofloxacin, Ketoconazole, DTNB; 5,5-dithiol-bis-(2-nitrobenzoic acid) also known as Ellman’s reagent, Tryptic Soy Broth (TSB), *P*-iodonitrotetrazolium violet (P-INT) dye, DCM (Dichloromethane).

Plant Extracts Preparation

The plant parts (leaves, stems, and roots) of *Byrsocarpus coccineus* (FHI 113906) and *Ficus exasperata* (FHI 113907) were collected from Ipara Remo, Remo North, Ogun State, Nigeria, and Amina way, University of Ibadan, Ibadan, Oyo State, Nigeria respectively. Authentication was carried out by Mr Edunjobi at the Forest Herbarium, Ibadan, Oyo State. The powdered plant material was extracted with absolute methanol for 72 hours. Extracts were filtered and concentrated using rotary evaporator.

Plant-chemical Screening

Plant crude extracts were screened to determine the availability of secondary metabolites including saponins, coumarins, phenols, flavonoids, alkaloids, steroids, and anthraquinones [9, 10].

Brine Shrimp Lethality Assay

By dissolving sea salt in 40 g/L of distilled water and adding 6 mg/L of dried yeast, artificial seawater was created. Eggs of brine shrimp (*Artemia salina*) were incubated in artificial seawater for 48 hours at 27 °C in a warm room. The *nauplii* of brine shrimp were gathered and separated from the eggs. In triplicate, serial dilutions were prepared in 96-well microplate wells using 100 uL of seawater. Seawater from negative control wells also had 0.5% dimethyl sulfoxide (DMSO). Each well of the covered micro-well plate received about 100 uL of seawater containing 10 brine shrimp *napulii* larvae, which were then incubated for 24 hours at 27°C. The quantity of dead brine shrimp *nauplii* in each well was then counted after the plates were inspected under a binocular microscope. To immobilize the brine shrimp *napulii*, 100 µL of methanol was added to each well, and the total number of shrimps in each well was counted.

The LC_{50} (µg/mL) was determined via *Eqn 1*:

$$\%\ Lethality = \frac{Number\ of\ dead\ nauplii \times 100}{Total\ Number\ of\ nauplii} \quad Eqn\ 1$$

This study decided to have a preview of the toxicity level of these plant extracts; hence, the need for the Brine shrimp lethality assay. This assay explains the level of toxicity of an extract despite its activity for the biological assays.

***In vitro* Acetylcholinesterase Inhibitory Assay**

Carried out according to a modified Ellman’s method described by Obuotor, 2004 [11, 12]. 20 µL of 10 mM DTNB (Ellman's reagent- [5,5l-dithiobis-(2-nitrobenzoic acid)]), 20 µL of the substrate (25 mM ATChI), and 240 µL of phosphate

buffer (pH 8.0) were prepared, mixed, and incubated for 30 minutes at 37 °C. The reaction mixture included 20 µL of the enzyme (Acetylcholinesterase-ATCh) in triplicate.
Eserine (positive control) was also serially diluted to obtain the final concentrations using the two-fold dilution factor. By monitoring the change in absorbance per minute brought on by the creation of the 5-thio-2-nitrobenzoate anion, the rate of hydrolysis of the substrate was ascertained spectrophotometrically at a wavelength of 412 nm every 30 seconds for 4 minutes. Without the extracts, there was enzyme activity in the negative control.
Eqn 2 was used to determine the % cholinesterase inhibition:

$$\%\ AChE\ Inhibition\ (I) = \frac{(\Delta a - \Delta b)}{\Delta a} \times 100 \quad Eqn\ 2$$

Where; I (%) = Percentage inhibition,
Δa = Variation in the negative control's absorbance (enzyme activity without extract)

Δb = Variation in extract absorbance (enzyme activity when extract is present).

The IC_{50} value, represented as the mean ± standard deviation of three duplicate values, was used to express the final activity in terms of the concentration that resulted in 50% inhibition of the AChE enzyme.

***In vitro* Antimicrobial Assay (Broth Micro-dilution Method)**
To create a stock solution of 10 mg/mL, the samples were dissolved in methanol. A concentration range of 200-0.75 µg/mL was achieved by serially diluting each sample (from the stock solution) in the microplate wells. For the antibacterial and antifungal tests, the standard medications were ciprofloxacin (10 µg/mL) and ketoconazole (10 µg/mL), respectively, in Tryptic Soy Broth (TSB) medium. Diluting the reference medications yielded a range of 10–0.1325 µg/mL. The test microorganism was diluted to 0.5 McFarland standard (1.0 x 106 CFU/mL-CFU-Colony Forming Units) from its overnight culture. 20 µL of the test microorganisms were added to each well that contained 100 µL of the different extract concentrations. The wells were then incubated for 24 hours (for bacteria) and 48 hours (for fungi) at 37°C. Following incubation, each well received 20 µL of 0.2 mg/mL P-iodonitrotetrazolium violet (P-INT), which was then incubated for an additional half hour. A shift in color from violet to red formazan was indicative of microbial growth, whereas clear or yellow coloring suggested no growth. Utilizing the microtitre plate method used in the MIC determination, the minimum bactericidal concentration (MBC) was further ascertained. The TSB in the microtitre plate wells that did not exhibit any growth following incubation during MIC testing was streaked independently on tryptic soy broth and cultured for 48 hours at 37°C, inverted. After 24 to 48 hours of incubation, the MBC was thought to be the lowest concentration of the extract that did not cause any growth on the TSB.

Data Analysis
Experiments were carried out in triplicate. Data was subjected to the analysis of One-way ANOVA using GraphPad Prism, showing a significant difference at $p<0.05$.

RESULTS

Plant-chemical Screening
Secondary metabolites including saponin, phenol, and alkaloid are present in *B. coccineus* and *F. exasperata* leaves, stems, and roots. Flavonoid is present in both plant extracts except in the root of *F. exasperata*. Coumarin was found present in both plant extracts except in the stem of *F. exasperata*. Anthraquinones are found in *B. coccineus* roots, while steroids are absent in all extracts

Assay for Brine Shrimp Lethality
FEL, BCS, FER and BCR are highly toxic (LC_{50} 31.62±2.83 µg/mL, 67.87±4.24 µg/mL, 74.23±6.36 µg/mL and 88.84±3.54 µg/mL, respectively), while the methanol extracts of BCL and FES are moderately toxic (LC_{50} 103.4±4.24 µg/mL and 173.3±3.54 µg/mL, respectively) at $p<0.05$.

Acetylcholinesterase Inhibitory Assay
BCR and FES showed the highest percentage inhibition (97.4 ± 0.80% and 91.6 ± 1.30%) at 5 mg/mL respectively, while BCS had a dose-dependent concentration inhibition of AChIEl (1.25 mg/mL - 0.16 mg/mL) at $p<0.05$.

The inhibition of AcChEl by Eserine (Standard drug) in dose-dependent concentrations at $p<0.05$ (Table 4).

Assay for Antimicrobials
The lowest minimum inhibitory concentration against *Staphylococcus aureus* and *Klebsiella pneumoniae* occurred at 12.5 µg/mL and 25 µg/mL, *Trichophyton rubrum* and *Candida albicans* at 25 µg/mL, *Bacillus subtilis* and *Escherichia coli* at 50 µg/mL.

BCL, BCS, and BCR exhibited minimal inhibitory activity against *C. albicans* at all concentrations. FEL and FER exhibited the highest inhibitory activity at 100 µg/mL and 50 µg/mL, respectively. BCL, BCS, and BCR completely inhibited *T. rubrum* growth at 200 µg/mL.

DISCUSSION

This study identifies bioactive phytoconstituents in plants through phytochemical screening [13]. The results obtained align with previous studies on *Byrsocarpus coccineus* root

Table 1: Plant-chemical Screening of *Brysocarpus coccineus* and *Ficus exasperata*

Test	Methanol Crude Extract of Plant Samples					
	BCR	**BCS**	**BCL**	**FER**	**FES**	**FEL**
Saponins	Present	Present	Present	Present	Present	Present
Coumarins	Present	Present	Present	Present	Absent	Present
Phenols	Present	Present	Present	Present	Present	Present
Flavonoids	Present	Present	Present	Absent	Present	Present
Alkaloids	Present	Present	Present	Present	Present	Present
Steroids	Absent	Absent	Absent	Absent	Absent	Absent
Anthraquinones	Present	Absent	Absent	Absent	Absent	Absent

Key: BCR-*Byrsocarpus coccineus* Root Extract, **BCS-***Byrsocarpus coccineus* Stem Extract, **BCL-***Byrsocarpus coccineus* Leaf Extract, **FER**-*Ficus exasperata* Root Extract, **FES**-*Ficus exasperata* Stem Extract, **FEL**-*Ficus exasperata* Leaf Extract.

Table 2: Brine Shrimp Lethality Assay of *Byrsocarpus coccineus* and *Ficus exasperata*

Samples	LC_{50} (µg/mL)
BCR	88.84±3.54
BCS	67.87±4.24
BCL	103.4±4.24
FER	74.23±6.36
FES	173.3±3.54
FEL	31.62±2.83
DOX	10.90±0.71

Key: BCR-*Byrsocarpus coccineus* Root Extract, **BCS-***Byrsocarpus coccineus* Stem Extract, **BCL-***Byrsocarpus coccineus* Leaf Extract, **FER**-*Ficus exasperata* Root Extract, **FES**-*Ficus exasperata* Stem Extract, **FEL**-*Ficus exasperata* Leaf Extract, **DOX**-Doxorubicin (Standard drug).

Table 3: AChEI Assay Result of *Byrsocarpus coccineus* and *Ficus exasperata*

Conc. (mg/mL)	Average Inhibition (%)			
	BCR	**BCS**	**FER**	**FES**
5.00	97.4 ± 0.80	76.0 ± 0.20	66.7 ± 0.60	91.6 ± 1.30
2.50	70.7 ± 0.50	76.3 ± 0.60	54.3 ± 0.70	78.8 ± 1.00
1.25	60.5 ± 0.40	62.5 ± 0.60	50.6 ± 0.60	47.6 ± 0.70
0.63	52.2 ± 0.50	54.1 ± 0.50	39.0 ± 0.70	37.5 ± 0.80
0.31	36.2 ± 0.90	44.4 ± 0.40	37.6 ± 0.70	23.4 ± 0.90
0.16	29.8 ± 0.20	39.0 ± 0.40	35.2 ± 0.60	7.1 ± 0.30

Key: BCR-*Byrsocarpus coccineus* Root Extract, **BCS-***Byrsocarpus coccineus* Stem Extract, **FER**-*Ficus exasperata* Root Extract, **FES**-*Ficus exasperata* Stem Extract

Table 4: AChEI Assay Results of Eserine (Standard drug)

Concentration (mg/mL)	Average Inhibition (%)
0.1	69.8
0.05	59.7
0.025	44.3
0.0125	35.5
0.00625	29.3
0.003125	15.0

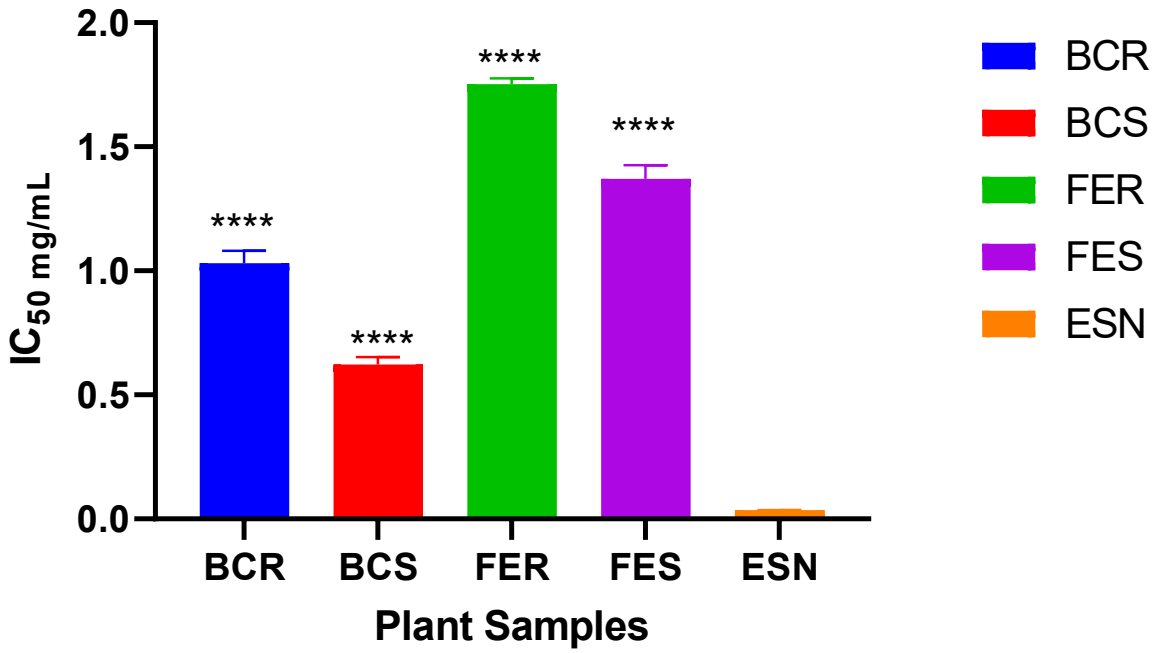

Figure 1: IC_{50} (mg/mL) of the Anticholinesterase Assay of *Byrsocarpus coccineus* and *Ficus exasperata*
Brine Shrimp Lethality Assay of *Brysocarpus coccineus* and *Ficus exasperata* exhibiting significant AChEI activity at p<0.05. *= Significant difference in relation to standard drug (*= P < 0.05, ****= P < 0.0001). **Key: BCR-***Byrsocarpus coccineus* Root Extract, **BCS-***Byrsocarpus coccineus* Stem Extract, **FER**-*Ficus exasperata* Root Extract, **FES**-*Ficus exasperata* Stem Extract, **ESN-**Eserine (Standard drug)

Table 5: Minimum Inhibitory Concentration (MIC) Values of *B. coccineus* and *F. exasperata* in µg/mL

Bacteria	BCL	BCS	BCR	FEL	FES	FER	CIP/KCZ
Staphylococcus aureus	200	200	200	12.5	200	25	0.78
Bacillus subtilis	100	100	200	50	100	-	0.78
Klebsiella pneumonia	50	50	50	50	25	25	0.78
Escherichia coli	100	100	200	100	100	50	0.78
Fungi							
Candida albicans	-	200	-	-	25	50	0.78
Trichophyton rubrum	100	100	100	50	100	50	0.78

Key: BCR-*Byrsocarpus coccineus* Root Extract, **BCS-***Byrsocarpus coccineus* Stem Extract, **BCL-***Byrsocarpus coccineus* Leaf Extract, **FER**-*Ficus exasperata* Root Extract, **FES**-*Ficus exasperata* Stem Extract, **FEL**-*Ficus exasperata* Leaf Extract, **CIP-** Ciprofloxacin and **KCZ-** Ketoconazole

Table 6: Values for the Minimum Fungicidal Concentration (MFC) and Minimum Bactericidal Concentration (MBC) of *B. coccineus* and *F. exasperate.*

Minimum Bactericidal Concentration (µg/mL)							
Organisms/ Sample	BCL	BCS	BCR	FEL	FES	FER	CIP/KCZ
Staphylococcus aureus	-	-	-	-	-	200	1.56
Bacillus subtilis	-	-	-	100	200	50	1.56
Klebsiella pneumoniae	100	100	100	-	100	100	1.56
Escherichia coli	-	-	-	-	200	100	1.56
Minimum Fungicidal Concentration (µg/mL)							
Candida albicans	-	-	-	100	50	100	1.56
Trichophyton rubrum	200	200	200	-	-	100	1.56

Key: BCR-*Byrsocarpus coccineus* Root Extract, **BCS-***Byrsocarpus coccineus* Stem Extract, **BCL-***Byrsocarpus coccineus* Leaf Extract, **FER**-*Ficus exasperata* Root Extract, **FES**-*Ficus exasperata* Stem Extract, **FEL**-*Ficus exasperata* Leaf Extract, **CIP-** Ciprofloxacin and **KCZ-** Ketoconazole

extracts, revealing the presence of flavonoids, alkaloids, saponins, anthraquinones, and phenols, and the absence of steroids in leaves [6, 14, 15].

Cytotoxicity screening test to determine the degree of toxicity of plant extracts *in vivo* is the brine shrimp lethality experiment [16]. Based on Clarkson's toxicity indices, a substance is considered non-toxic when its LC_{50} is greater than 1000 µg/mL; weakly harmful at 500 µg/mL < LC_{50} < 1000 µg/mL; moderately toxic with 100 µg/mL < LC_{50} < 500 µg/mL and highly harmful at LC_{50} < 100 µg/mL [17]. The result from this study aligns with the research carried out by Ogundare *et al.,* [18].

Plant extracts significantly inhibited acetylcholinesterase in a way that depends on concentration, with the average percentage inhibition values decreasing as the extract concentration increased [19]. *In vitro* acetylcholinesterase inhibitory assay was carried out on the stem and root of the selected plants as the leaves of *Ficus exasperata* have been explored for this activity in a study carried out by Omeje *et al.,* 2020 [23]. Although there has been limited research on the acetylcholinesterase inhibitory activity of Byrsocarpus *coccineus,* this study researched the effects of *B. coccineus* and *F. exasperata* stem and root as a foundation for the comparison of results obtained. Hence, future research could explore the potential of *Byrsocarpus coccineus* leaves on inhibiting acetylcholinesterase. The pattern of inhibition in this study is consistent with the standard drug Eserine. BCR and FES showed the highest percentage inhibition of the enzyme AChE at 5 mg/mL, while BCS was consistent in inhibiting it in the remaining concentrations. The efficacy of each extract was determined by evaluating their respective IC50 values, which are the maximal inhibitory concentration to inhibit 50% of the enzyme's action [20]. *B. coccineus* stem had the highest acetylcholinesterase inhibiting activity at 0.622 ± 0.03 mg/mL, followed by its root at 1.03 ± 0.05 mg/mL. *F. exasperata* root and stem showed lower AChE inhibiting activity at 1.753 ± 0.023 mg/mL and 1.37 ± 0. 056 mg/mL, in contrast to serine, respectively. The differences in their inhibiting potential may be due to variations in the quantity and composition of natural AChE inhibitors [21].

The broth micro-dilution method determines plant extracts' minimum inhibitory concentrations (MIC) by estimating the concentration of the tested antimicrobial agent in the broth and the area of no visible growth against the microbial isolate [22]. The MIC of the extracts was 200 µg/mL against the six microorganisms. The results from this study align with earlier research conducted [15, 19] with slight variations that could be due to differences in the extraction methods and solvents [15] conducted an antimicrobial study using agar well diffusion to inhibit *S. aureus* and *E. coli*. Ajala *et al.* [19] used agar cup diffusion to show *Ficus exasperata* leaves showed antibacterial activity against *Staphylococcus aureus, Escherichia coli*, *Bacillus subtilis* and *Klebsiella pneumonia*. The plants exhibited higher zones of inhibition against *Candida albicans*. However, the results did not align with the ranking of the bacterial pathogens.

BCL, BCS, and BCR had minimal inhibition against *S. aureus* (<100% inhibition), *B. subtilis*, and *E. coli* at all concentrations, except for *K. pneumoniae*, which was completely inhibited (100% inhibition) by the three extracts at 100 µg/mL. *S. aureus* was not completely inhibited (<100% inhibition) by FEL, FES, and FER throughout all the concentrations. *B. subtilis* was totally suppressed (less than 100% suppression) by FEL at 100 µg/mL, FES at 200 µg/mL, and FER at 50 µg/mL. FES and FER completely inhibited (100% inhibition) *K. pneumoniae* at 100 µg/mL, while FEL showed no complete inhibition (<100% inhibition) at all concentrations. *E. coli* was completely inhibited at 200 µg/mL and 100 µg/mL and showed no complete inhibition by FEL at all concentrations.

CONCLUSION

The stems and roots of *Byrsocarpus coccineus* and *Ficus exasperata* exhibited acetylcholinesterase-inhibiting potential in a concentration-dependent manner. However, *Byrsocarpus coccineus* stem exhibited the highest anticholinesterase activity, which can serve as a potential template for providing alternative management in the treatment of memory loss. The leaves, stems, and roots of both plants revealed variations in their inhibiting potentials to the growth of *Candida albicans, Escherichia coli, Klebsiella pneumoniae, Bacillus subtillis, Staphylococcus aureus,* and *Trichophyton rubrum*, which may be attributed to the similarity and dissimilarity of the phytochemical constituents present in these medicinal plants.

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AUTHORS' CONTRIBUTION

All authors have contributed to the content of this manuscript, accepted responsibility for its entire content, and consented to its submission to the journal.

CONFLICT OF INTEREST

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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