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Phytochemical Constituents and Biological Activity of Fractions of Stem and Leaves of *Acalypha inferno*

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Authors' contributions

This work was carried out in collaboration between all authors. Authors CL, JNND and JJT designed the study, performed the statistical analysis and wrote the first draft of the manuscript. Authors JNND and DT performed the phytochemical and antioxidant assays. Authors FCMR and CYA performed the antimicrobial assays. Authors JNND and JJT managed the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

Aims: *Acalypha inferno* is an ornamental plant which has been shown to be a phytoremediator of zinc. Other species of this genus used traditionally have been proven to possess pharmacologically active constituents. The study sought to evaluate the phytochemical constituents, in vitro antioxidant activity and antimicrobial effect of *Acalypha inferno* crude extracts and fractions of leaves and stem.
Place and Duration of Study: Study was conducted in the Department of Biochemistry and Biotechnology and the Central Laboratory of the Kwame Nkrumah University of Science and Technology, Kumasi Ghana between October 2016 and May 2017.
Methodology: Phytochemical constituents, DPPH assay, total phenolic content and antimicrobial

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effect were analyzed using standard methods. FT-IR and UV-VIS spectroscopy were used to identify functional groups and major constituents. Heavy metals in the plant were evaluated by wet digestion of sample and measurement using AAS.
Results: Phytochemicals included triterpenoids, sterols, alkaloids, coumarins, flavonoids, saponins, glycosides and hydrolysable tannins. The Total Phenolic Content (TPC) of the leaf extract was 1.818 mg/ml ± 0.062 (EC_{50} = 0.03995 mg/ml ± 0.00084). The FTIR revealed the presence of alcohols, amines, aromatic compounds, amides, carboxylic acids, esters and alkynes. Iron and zinc were also detected. The stem and leaf fractions exhibited antimicrobial activity against *S. typhi*, *P. aeroginosa* and *S. aureus.*
Conclusion: *Acalypha inferno* is rich in antioxidants and bioactive compounds which exhibit antimicrobial activity against some bacteria.

Keywords: Acalypha inferno; phytochemicals; antioxidants; FTIR and UV-VIS spectrophotometry.

1. INTRODUCTION

For centuries now, plants have been used for the treatment of several ailments before the evolution of orthodox drugs which have their active components synthesized from plants with modifications [1]. Plants are known to produce various bioactive molecules making them a rich source of medicines. Secondary plant metabolites have been investigated as a source of medicinal agents with anti-microbial and anticancer properties [2].

Oxygen metabolites can be highly toxic. Reactive oxygen species (ROS) are known to cause many cell disorders and the development of many diseases [3]. Antioxidants inhibit oxidative processes thus have essential physiological roles in the body [4]. The antioxidant properties of plants have been associated with the presence of phenolic compounds [5]. Almost all medicinal plants have been shown to contain enormous amounts of antioxidants such as polyphenols. The neutralizing ability of antioxidants in cells to scavenge free radicals and reactive oxygen species is vital in reducing cell disorders, cancers and tissue damage and the occurrence of several human diseases [6]. The most predominant source of natural antioxidants has been fruits and vegetables as well of medicinal trees and herbs. The over-reliance on the natural ecosystem has also been a worry for conservationist and may lead to the extinction of the very useful plants including *Taxus brevifolia* and *Podophyllum peltatum* [7]. Researches has shown that most underutilized plants including weeds and ornamental plants could have medicinal benefits [8]. The plant under study is *Acalypha inferno*.

Acalypha inferno has little or no reported pharmacological properties. However, members of the genus *Acalypha* are known to be pharmacologically active [9,10]. According to a study conducted by Abioye et al. [11], *Acalypha inferno* showed ability to remediate zinc (Zn) in contaminated soil. The leaves accumulated the highest concentration of zinc followed by stem and roots. This study therefore sought to characterize the phytochemical constituents of hydro-ethanolic extract of the stem and leaves of *Acalypha inferno* and solvent fractions as well as assess its biological activities including free radical scavenging ability and antimicrobial effect on clinical isolates.

Fig. 1. *Acalypha inferno* whole plant

2. MATERIALS AND METHODS

2.1 Plant Preparation and Extraction

The stem and leaves of *Acalypha inferno* were collected from the forecourts of Biochemistry Annex in October, 2016 before 9:00 am, washed, shade dried and milled. The plant material was authenticated at the Department of Pharmacognosy, KNUST-Kumasi by Dr. George

Sam (taxonomist) and a voucher specimen deposited at the herbarium for reference. The 50% hydro-ethanolic extract was prepared by dissolving 100 g of the sample into 1000 ml of 50% hydroethanol (50:50 v/v ethanol: water). The mixture was left on a mechanical shaker for 24 hours at room temperature. The mixture was filtered and concentrated using a Heidoph Rotary Evaporator (Germany) at 60°C under reduced pressure. Plant extracts were freeze dried to obtain the powdered form of both the leaf and the stem crude extracts.

2.2 Sequential Fractionation of Plant Extracts

The plant extracts were subjected to sequential fractionation with petroleum ether, ethyl acetate and methanol in an increasing order of solvents' polarities. Thirty grams of the crude stem and leaf extracts of *A. inferno* were weighed, transferred into separating funnel and 300 ml of the solvent added. After vigorous shaking, the set-up was left for 48 hours and the extracted portion was drained out. The residue was dried to evaporate all the solvent present after which it was poured into the separating funnel to be extracted using the subsequent solvent. The remaining residue after the fractionation was labelled as the hydro fraction. The four fractions obtained were concentrated for further analysis. The yields of the fractions were calculated.

2.3 Phytochemical Screening

The presence of glycosides, saponins, tannins, alkaloids, flavonoids, triterpenoids, coumarins, and sterols were tested for using standard chemical methods as described by Harborne [12], Rahilla et al. [13] and Sofowora [14].

2.4 Heavy Metal Analysis

About 1 g of each of the extracts and the raw samples were weighed into a 50 ml digestion tube. All the samples were wet digested using 1 ml of H_2O, 2 ml of HCl, 5 ml of 1:1 HNO_3:$HClO_4$ and 2 ml of H_2SO_4 for 20 minutes. At a temperature of 150°C the samples were heated in a digestion block. The digested samples were allowed to cool after which they were diluted with 50 ml of distilled water. The digests were analyzed to determine the levels of heavy metals in the samples. The heavy metals analyzed were lead, copper, cadmium, nickel, zinc and iron using the Atomic Absorption Spectrometer (Varian AA 240FS) utilizing a long path air acetylene burner and cathode lamp for respective metals.

2.5 Determination of Total Phenols

The total phenolic content of the plant extracts was determined using the Folin Ciocalteau assay with a few modifications [15]. Stock solutions of 5 mg/ml of the crude extracts and the gallic acid standard were prepared. Two-fold serial dilutions were carried out on the crude extracts and gallic acid to obtain various concentration ranges of 2.5-10 mg/ml and 0.15625-5 mg/ml respectively. A volume of 10 µL of the plant extracts was aliquoted into Eppendorf tubes and diluted with 790 µL of distilled water. A volume of 50 µL of the Folin Ciocalteau reagent was added to each of the diluted samples and the resulting solution was incubated in the dark for 8 minutes after which 150 µL of 7% of Na_2CO_3 (sodium carbonate) was added. The mixture was incubated again for 2 hours in the dark at room temperature. The absorbance of the resulting solutions was read at a wavelength of 750 nm using a microplate reader (Synergy H1 Reader, USA). Triplicate measurements were performed. The results were expressed in milligrams of gallic acid equivalents per dry mass of the extract (mg of Gallic Acid/DM).

2.6 Determination of Antioxidant Activity

The antioxidant activity of the stem and leaf extract was examined using scavenging effect on the stable DPPH free radical [16]. The plant extracts were diluted using two-fold dilution to obtain concentrations from 0-10 mg/ml. 0.05 mM DPPH was prepared with methanol as blank. A volume of 100 µl of DPPH was added to 100 µl of each extract. Ascorbic acid was used as the standard with concentrations ranging from 0-10 mg/ml and distilled water as the blank. Negative controls were included. After 20 minutes of incubation the absorbance was measured using a microplate reader (Synergy H1, USA) at a wavelength of 517 nm. Triplicate experiments were performed. The radical scavenging activity of extracts were expressed as percentage of inhibition using the formula:

$$\text{Percent (\%) inhibition of DPPH activity} = \frac{A_B - A_A}{A_B} \times 100$$

where A_A and A_B represent the absorbance values of the test and of the blank, respectively [17]. A graph of percentage inhibition versus

concentration was used to determine the effective concentration at 50% inhibition (EC_{50}).

2.7 UV-Vis Analysis and Fourier Transform Infrared (FTIR) Spectrophotometric Analyses

The fractions of both stem and leaf (10 mg/ml) were diluted in a ratio 1:10 using respective solvents and analyzed at a wavelength ranging from 200 – 450nm. A double beam Ultraviolet-visible spectrophotometer (Perkin Elmer, USA) was used to detect the characteristic peaks present in that range. The peaks showing the maximum wavelength was recorded. The FTIR analysis was performed on the fractions to determine the functional groups present. The FTIR (Perkin Elmer, USA) detected characteristic peaks which represented the functional groups.

2.8 Antimicrobial Activity Profiling

2.8.1 Preparation of culture media

Nutrient Agar (Oxoid, CM0003, England), Bacteriological Peptone (Sigma-Aldrich, P0556, Germany), and Mueller-Hinton agar (Oxoid, CM0337, Oxoid Ltd, England) were used. The media were prepared according to manufacturer's instruction. Chloramphenicol (Oxoid, CT00143 Oxoid Ltd, England) and DMSO (Sigma, D5879, Germany) were also used.

2.8.2 Inoculum preparation

The stock cultures of *Pseudomonas aeroginosa, Escherichia coli, Salmonella typhi* and *Staphylococcus aureus* were sub cultured onto fresh nutrient agar plates to obtain pure colonies. Single colonies of each organism were suspended in test tubes containing 5 ml of sterilized bacteriological peptone and incubated at 37°C for 10-18 hours to attain the turbidity of 0.5 McFarland standards. The turbidity of the actively growing broth cultures which were not in conformity to the standard (exceeded the standard) were adjusted with sterile bacteriological peptone to obtain turbidity optically comparable to that of 0.5 McFarland Standard (approximately 1-2 x 10^8 CFU/ml for *E. coli* ATCC 25922) [18].

2.8.3 Preparation of plant fractions for the bioassays

Considering the preparation of stock solutions for the bioassays, 100 mg/ml, 200 mg/ml, and 300 mg/ ml of the fractionated plant extracts were reconstituted using 20% ^{v}/v DMSO (Sigma, D5879, Germany) resulting in percentage concentrations of 10%, 20% and 30% for the determination of the antimicrobial activity. The crude extract was dissolved in sterile distilled water for the aqueous extract preparation of same concentrations. The stock solutions were stored in a refrigerator until needed.

2.8.4 Antimicrobial activity assay (agar diffusion method)

The antimicrobial activity was carried out using the agar diffusion method [18,19]. The inoculums were inoculated by swabbing unto sterile Mueller-Hinton agar. A sterilized cock borer of an internal diameter of about 4 mm was used to punch six holes in the medium. The plant extracts were dispensed into the holes to a volume of 100 µL. The positive control was an antibiotic disc of 30 µg chloramphenicol and 20% v/v DMSO was used as negative control in a triplicate fashion. The plates were refrigerated for about 4 hours to allow for absolute diffusion of the extract and incubated at 37°C for 24 hours, after which the diameter of each zone of inhibition was measured in millimeters (mm) with a sterilized ruler.

2.9 Statistical Analysis of Results

One-way analysis of variance (ANOVA) was used for the data analysis. The means were assessed by Tukey's test at 5% significance level ($p < 0.05$) using Graph Pad Prism version 6.0. The results were expressed as mean ± standard deviation for triplicate measurements.

3. RESULTS

3.1 Extraction Yield

Hydroethanolic extraction of *A. inferno* yielded 11.5% for leaves and 4.00% for stem. Solvent fractions yielded various amounts of the initial 30 g for leaves and 20 g for stem extract (Table 1).

3.2 Phytochemical Constituent

Table 2 shows the phytochemical constituent of the raw and crude hydroethanolic leaves and stem extracts of *A. inferno*. Tannins, saponins, glycosides, flavonoids, coumarins and triperpenes were present in both raw and crude extracts. Alkaloids were present in stem only while sterols were present in raw plant materials.

Table 1. Percentage yield of fractions obtained from the crude extracts of *A. inferno*

Solvent fraction	Yield from leaf extract (30 g)	Yield from stem Extract (20 g)
Petroleum ether	0.03 % (0.02 g)	3.50 % (0.70 g)
Ethyl acetate	1.73 % (1.04 g)	21.75 % (4.35 g)
Methanol	12.45 % (7.47 g)	19.60 % (3.92 g)
Hydro	85.78 % (51.47 g)	52.15 % (11.03 g)

Table 2. Phytochemical constituents of stem and leaves of *A. inferno*

Phytochemical	*A. inferno* raw leaf	*A. inferno* leaf extract	*A. inferno* raw stem	*A. inferno* stem extract
Tannins	+	+	+	+
Saponins	+	+	+	+
Glycosides	+	+	+	+
Alkaloids	-	-	+	+
Flavonoids	+	+	+	+
Coumarins	+	+	+	+
Sterols	+	-	+	-
Triterpenoids	+	+	+	+

Present (+) or Not detectable (-)

3.3 Heavy Metal Content in *Acalypha inferno*

Table 3 shows the mineral content of raw and leaves extract. Only iron and zinc were detected.

3.4 Total Phenolic Content (TPC)

The TPC of the stem and leaves were extrapolated from the standard gallic acid linear plot (y = 0.693x + 0.062, R2 = 0.9962). The leaf extract had a TPC of 1.82 ± 0.06 mg/ml, higher than stem extract (0.67 ± 0.06 mg/ml, p = 0.000).

3.5 Antioxidant Activity of *A. inferno*

The DPPH radical scavenging ability of *Acalypha inferno* was evaluated to determine the antioxidant activity of the plant. The hydroethanolic leaf extract had an EC_{50} value of 0.03995mg/ml, the stem crude extract had an EC_{50} value of 0.14 mg/ml and the standard ascorbic acid had an EC_{50} value of 0.12 mg/ml.

3.6 FTIR Spectra of Fractions of *A. inferno*

FTIR spectra fractions of *A. inferno* leaves and stem are as shown in Fig. 3 and 4. The figures show the peak numbers, percentage transmittance (%) and frequency/wavenumber (cm^{-1}) in the spectrum graph with their corresponding functional groups. All fractions contained alcohols, amines (primary, secondary and aliphatic amines), aromatic compounds, alkenes and alkynes. Phenols were present in all the fractions with amides in only the petroleum ether fraction. Nitro compounds and carboxylic acids were present in all the fractions except the petroleum-ether fraction. For stem fractions, primary and aliphatic amines were common. Alcohols were present in the ethyl-acetate and petroleum-ether fractions. Secondary amines and amides were present in all fractions except the petroleum-ether fraction.

3.7 UV-Vis Spectrophotometric Analysis

The UV-VIS spectroscopy for petroleum-ether leaf fraction was analyzed at 200nm to 450nm. For fractions of leaves extract, petroleum ether had a maximum absorption between 200 and 272 nm (10 peaks), ethyl acetate 208 to 275 nm (10 peaks), methanol 204 to 336 nm (10 peaks) and hydro fraction 204 to 340 nm (10 peaks). For stem extract, the maximum rank of absorption are petroleum ether 213 – 310 nm (7 peaks); ethyl acetate 228 – 326 nm (3 peaks); methanol 209 – 263 (3 peaks); and hydro 204 – 280 nm (7 peaks).

Table 3. Heavy metal content of plant materials and extract

Heavy metals	Concentration mg/L		
	***A. inferno* raw leaves**	***A. inferno* leaves extract**	***A. inferno* raw stem**
Iron	1.608 ± 0.0542	0.1009 ± 0.0050	1.373 ± 0.0307
Copper	BDL	BDL	BDL
Zinc	0.3066 ± 0.0094	0.3144 ± 0.0065	0.4268 ± 0.0124
Lead	BDL	BDL	BDL
Cadmium	BDL	BDL	BDL
Nickel	BDL	BDL	BDL

BDL – Below Detection Limit. Detection Limit – 0.00001 mg/L.

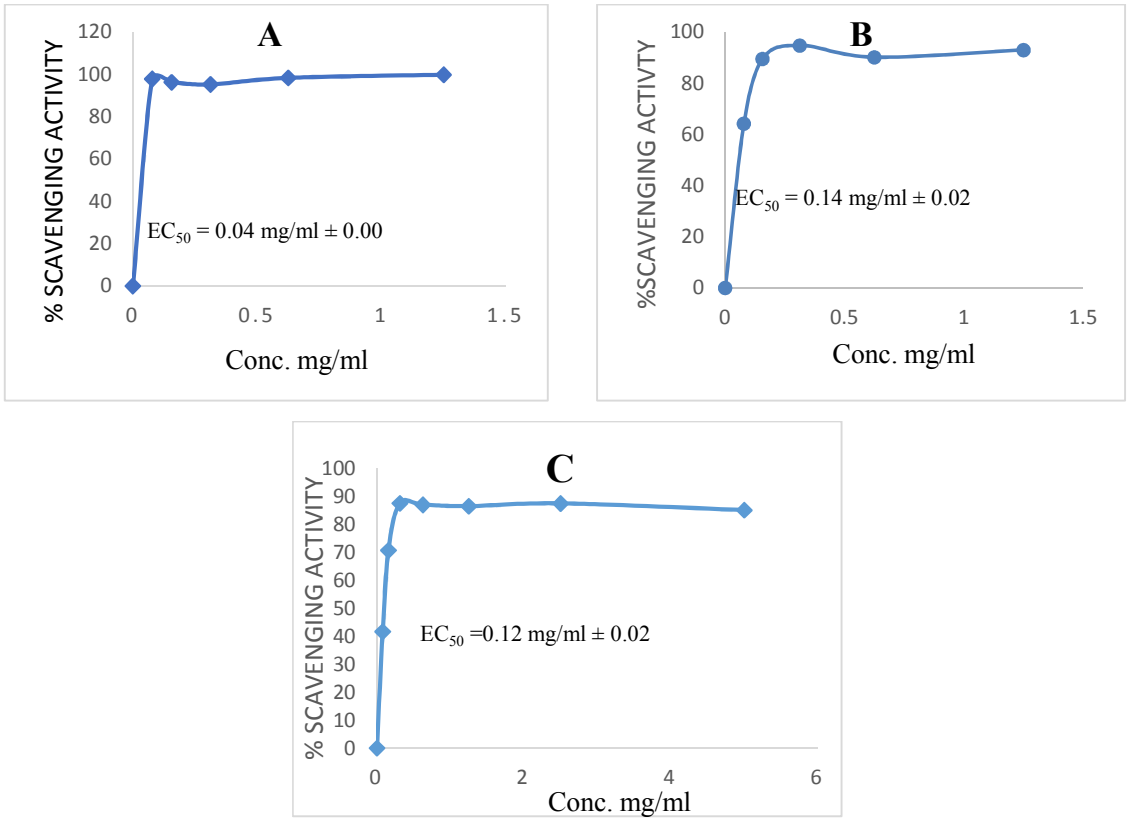
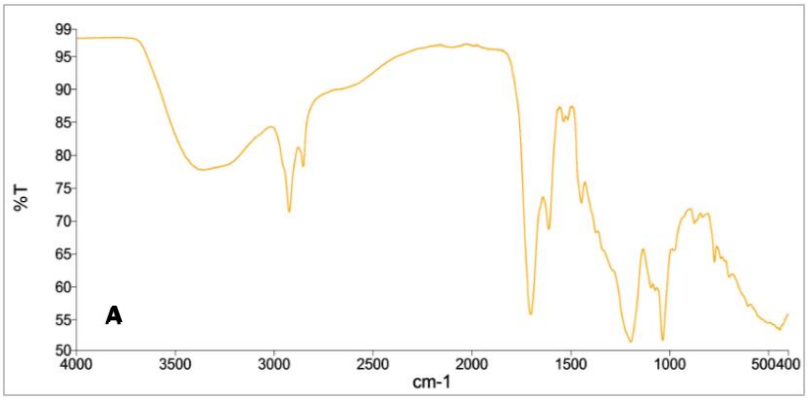


Fig. 2. Antioxidant activity of (A) *Acalypha inferno* leaf extract, (B) *Acalypha inferno* stem extract and (C) standard ascorbic acid



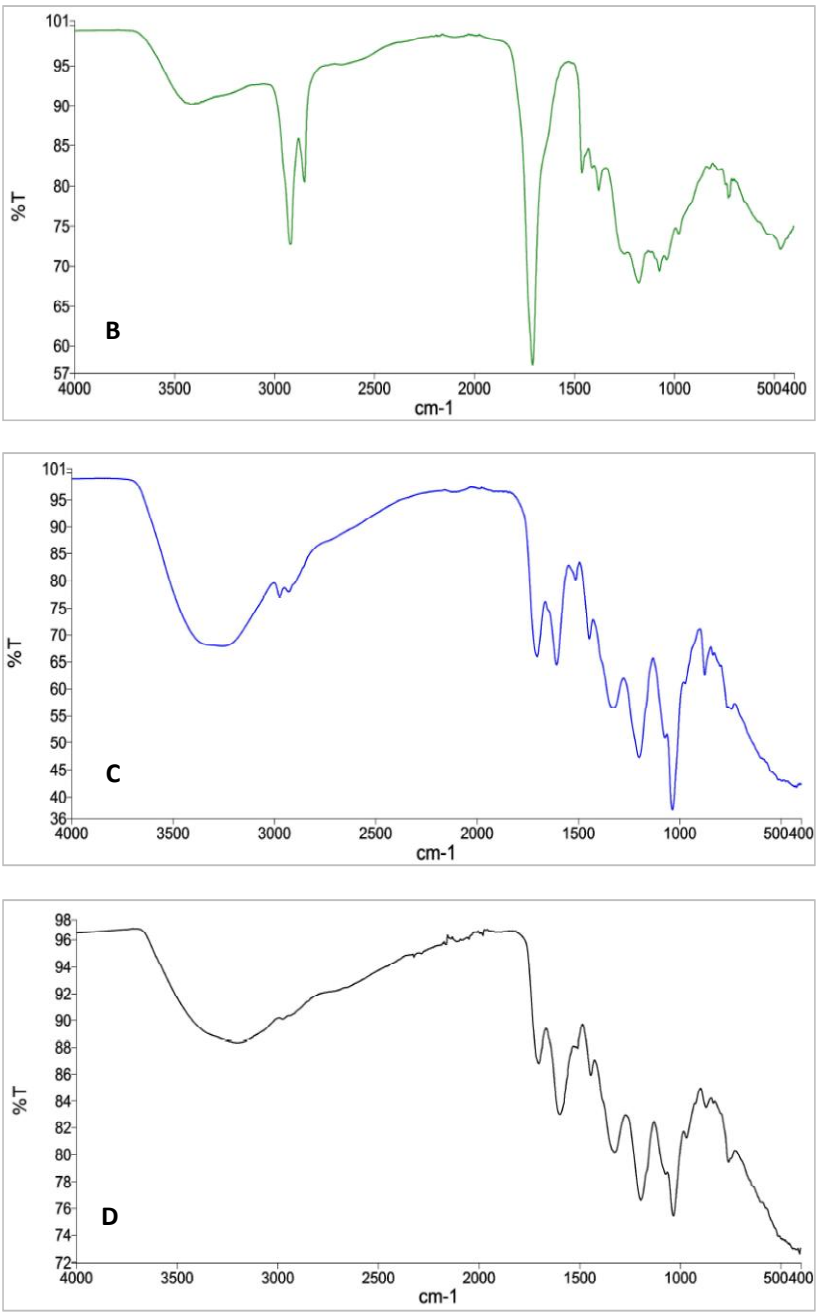


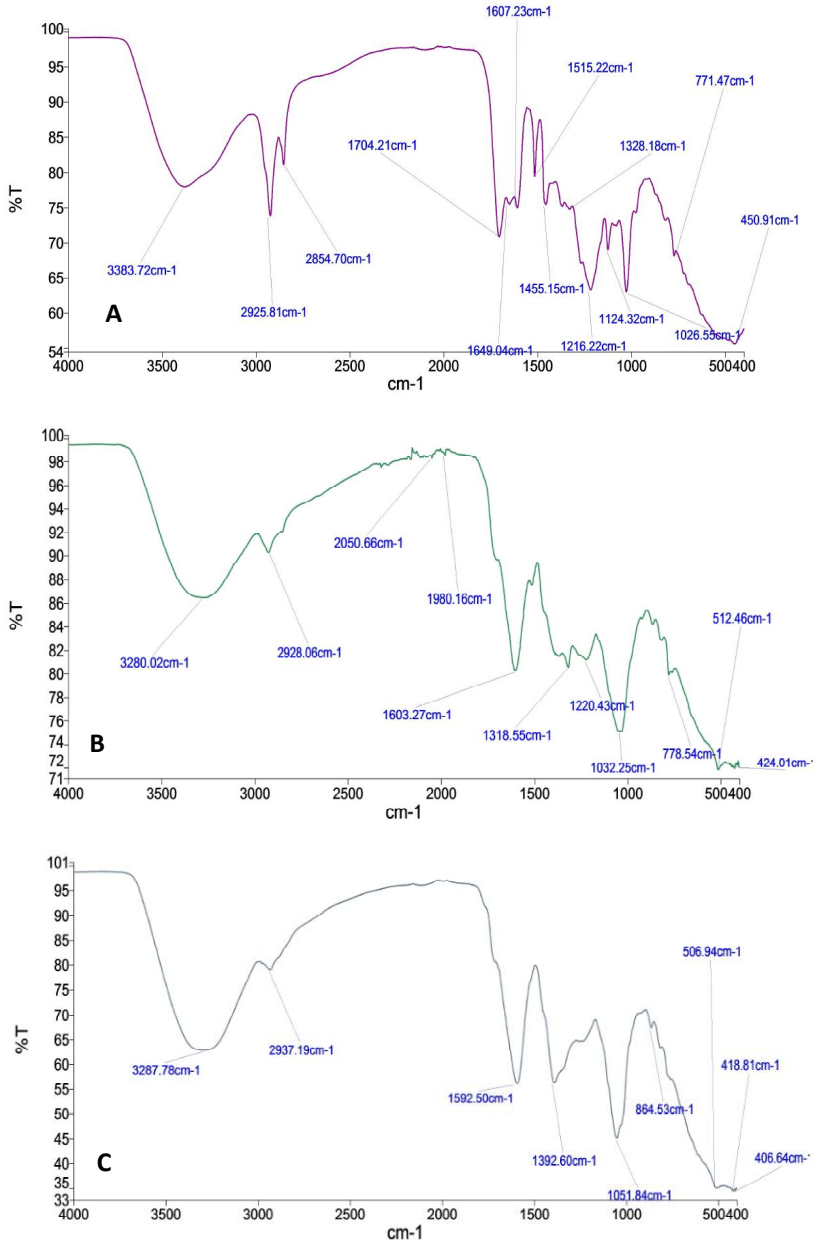
Fig. 3. FTIR spectrum of Petroleum-ether (A), Ethyl-acetate (B), Methanolic (C) and Hydro (D) fraction of *Acalypha inferno* leaves extract

3.8 Antimicrobial Activity

The results indicated some appreciable level of antimicrobial activity (zones of inhibition, 1.1 – 1.6 cm, $p<0.001$) in some fractions namely the crude stem extracts and ethyl acetate stem extract. The hydro and methanol extracts showed no antimicrobial prowess after the assay. The leaves however showed better antimicrobial prowess against the test organisms (zone of inhibition, 0.8 – 1.7 cm, $p<0.001$) except for *E. coli.* This notwithstanding, the methanol and hydro fractions did not exhibit any antimicrobial activity against any of the four test organisms (Fig. 4).

4. DISCUSSION

The crude extracts of leaves and stem as well as raw samples contained triterpenoids, saponins, coumarins, flavonoids, glycosides, sterols and hydrolysable tannins. These phytochemicals generally have medicinal and phytochemical activities [14]. Phenols and polyphenols (flavonoids, coumarins and tannins) have been shown to possess strong free radical scavenging properties [20]. In the current study, the stem and leaves of *A. inferno* showed effective antioxidant properties. The presence of these phytochemicals could account for the high antioxidant activity, predominantly in leaf extract. The crude extract of *A. inferno* leaf had an EC_{50} value of 0.03995 mg/ml which is more desirable compared with standard ascorbic acid and stem with EC_{50} values of 0.12441mg/ml and 0.14339 mg/ml respectively. Other species of the *Acalypha* genus such as *Acalypha fructicosa*, *Acalypha platyphylla*, *Acalypha siamensis*, *Acalypha guatemalensis* and *Acalypha wilkesiana* have also been shown to possess antioxidant properties [21-23].



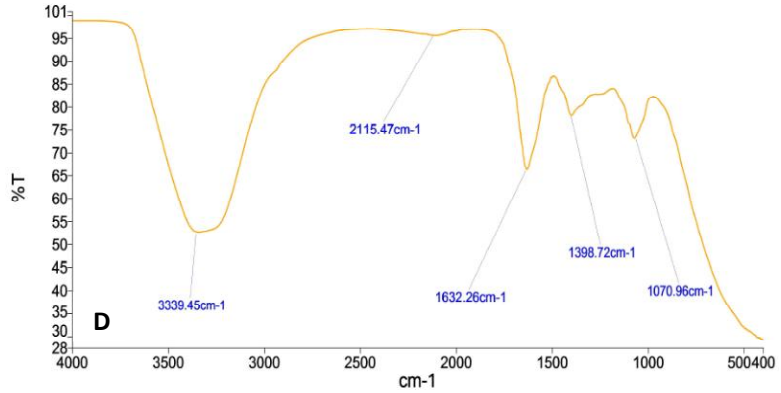


Fig. 4. FTIR spectrum of Petroleum-ether (A), Ethyl-acetate (B), Methanolic (C) and Hydro (D) fraction of *Acalypha inferno* stem extract

From Table 3, heavy metals including lead, cadmium, copper and nickel were below detection limits (0.00001 mg/L) for the raw leaf, raw stem and leaf crude extract. Iron was detected in the raw leaf, raw stem and leaf extract at concentrations of 1.608 mg/L, 1.373 mg/L and 0.1009 mg/L respectively. The iron concentration was highest in the raw leaf sample and lowest in the leaf extract but within permissible limits of 20 ppm in medicinal plants [24]. Iron is essential in the biosynthesis of haemoglobin and transport of oxygen and electrons throughout the body [25]. Iron deficiency could lead to myocardial infection, nose bleeding and gastrointestinal infections. In the analysis of zinc, it was found to be highest in the raw stem (0.4268 mg/L). Zinc concentrations in raw leaf and leaf extract were 0.3066 mg/L and 0.3144 mg/L respectively. All detected levels of Zn were within permissible limits (27.4 ppm) in edible plants [24,26]. Abioye et al. [11], reported *A. inferno* as a phytoremediator of zinc in contaminated soils. However, zinc detected was far below permissible limits. This could be due to low soil contaminants at site of sample collection. Zinc is essential in bone formation, wound healing, brain development and normal growth processes [26]. Deficiency of zinc in diabetics could lead to loss of touch and smell. The recommended dietary allowance (RDA) of zinc is 100 ppm [26]. Zinc toxicity symptoms include nausea, epigastric pain, vomiting and fatigue [27].

The FTIR analysis showed the presence of alcohols, phenols and aromatic compounds in petroleum-ether and methanolic fractions could account for total phenolic content of the stem. The higher total phenolic content in the leaf extract compared to the stem could be attributed to the presence of alcohols, phenols and aromatic compounds in all the fractions of the leaf extract.

A study by Karpagasundari and Kulothungan [28], showed that the occurrence of peaks ranging from 234-646 nm indicates phenolics and alkaloids. However, the UV-VIS analysis was done in a wavelength ranging from 200-450 nm. Flavonoids such as quercetin, kaempferol and isorhamnetin display maximum absorbance in a range of 250-260 and 370 nm. An absorption at 270 and 340 nm indicates the presence of flavonoids and its derivatives [29]. The flavonoids spectra usually consist of two absorption bands which range 230-290 and 300-350 nm. The relative intensities and positions of the peaks help in identifying the kind of flavonoids [30]. The FTIR spectrum confirms the presence of OH bonds which could be attributed to flavonoids and phenolic compounds. Chromophores like azo, carboxyl and nitrile groups could be present since they absorb maximally in the range 285-400 nm, 200-210 nm and 220-230 nm respectively. The UV-VIS spectra showed the presence of flavonoids, alkaloids, phenolics and some chromophores.

Some of phytochemicals are known to have antimicrobial tendencies or antibiotic potential. Typical of such are the flavonoids, triterpenoids and alkaloids which have been widely known to exhibit some antimicrobial properties against some bacteria [31]. The outcome of the study showed some antimicrobial activity in some fractions against some of the selected test organisms. This is attributed to the active phytochemical composition of the extracts [32,33].

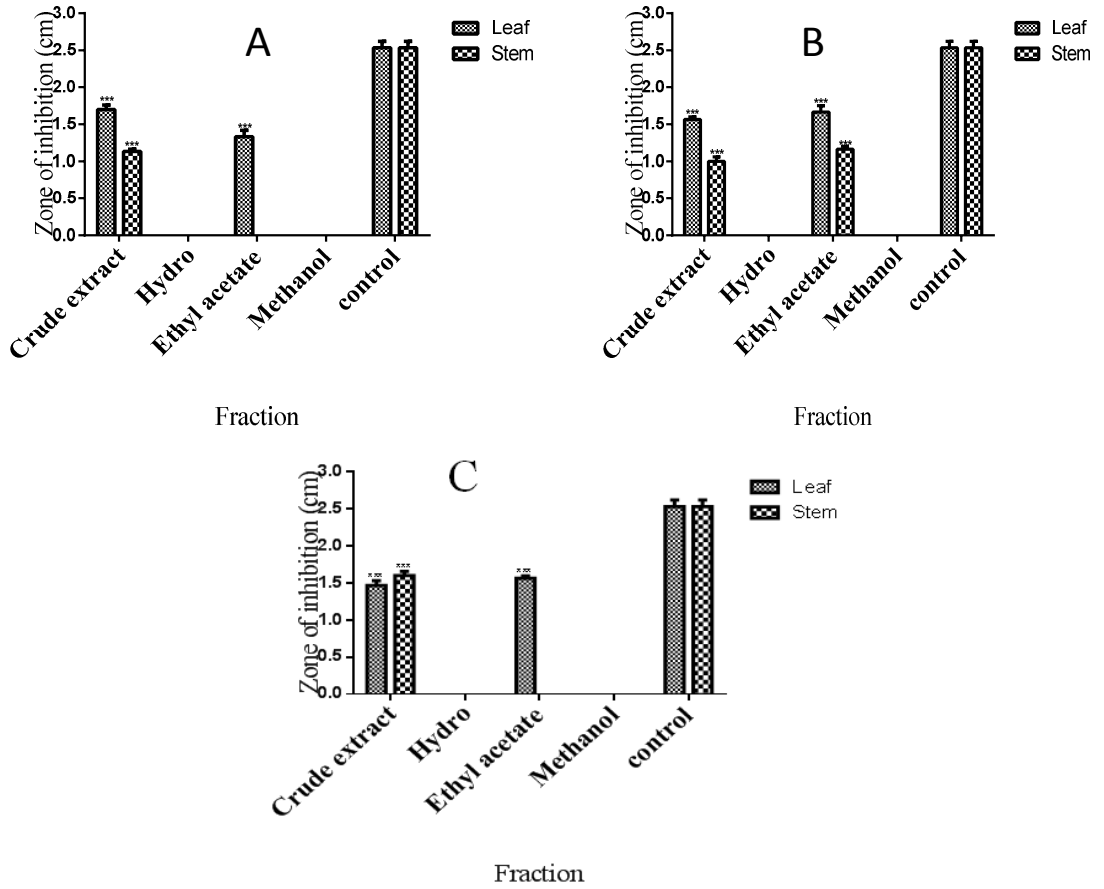


Fig. 5. Antimicrobial activity of stem and Leaf fractions of *A. inferno* against *P. aeroginosa* (A), *S. typhi* (B) and *S. aureus* (C) (*p<0.001 against Control)**

The phenomenon of interest was the significantly different antimicrobial activities recorded in the leaf and stem fractions. This could be due to the different distribution of phytochemicals at the different parts of a plant according to the prevailing conditions at the respective parts. Phytochemicals are produced mainly as secondary metabolites by plants especially when faced with harsh environmental conditions or pest and disease infestation as a mode of defense [34]. The functional group assay further highlights this observation as the leaf yielded almost twice the functional groups as observed in the stem. A similar observation was established in comparing the antimicrobial activity of various solvent fractions, where a significant difference in activity was observed (Fig. 5) which go to establish the different solubility of the phytochemicals in the different solvents [35]. The methanol and hydro fractions did not show any antimicrobial activity which establishes the relatively poor solubility of the phytochemicals in these solvents. This however does not suggest no phytochemicals were extracted into these solvents as the functional group study indicated some functional groups in these solvents. However, the challenge is rather with the concentration, thus with these solvents probably at higher concentrations (50%, 70%), they may exhibit the desired antimicrobial response [35] This is further emphasized by the phenomenon observed in the crude extract and ethyl acetate fraction of the stem where the lesser concentration (10%, 20%) yielded no activity but the higher concentration (30%) showed significant antimicrobial activity.

5. CONCLUSION

Acalypha inferno contains saponins, coumarins, triterpenoids, tannins, glycosides and flavonoids. *Acalypha inferno* leaf extract showed a higher antioxidant potential (ability to scavenge DPPH) compared to stem extract as well as antimicrobial

activity. The total phenolic content of the leaf extract was also higher than the stem. The results of the FTIR indicates the presence of alcohols, phenols, aromatic compounds nitro compounds, amines (primary, secondary and aliphatic amines), amides and carboxylic acids. The UV-VIS profile showed the peaks which represents flavonoids, phenolics, alkaloids and some chromophores. The levels of heavy metals Iron (Fe) and Zinc (Zn) detected in *Acalypha inferno* fall in the permissible range for medicinal plants. The study indicates that *Acalypha inferno* contains phytochemicals, antioxidants and bioactive compounds that have antimicrobial properties thereby making it a potential effective therapeutic agent.

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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