Antibiotic-potentiation, antioxidant, cytotoxic, anti-inflammatory and anti-acetylcholinesterase potential of *Antidesma madagascariense* Lam. (Euphorbiaceae)

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Highlights

• Acetone extract (AE) inhibited the growth of all tested ATCC and clinical isolate strains (MIC range: 0.25–4.00 mg/mL)
• Combination profiles of AE with commercial antibiotics revealed synergistic action against ATCC strain of *P. aeruginosa*
• Decoction and AE as well as its fractions showed significant antioxidant activity
• AE and its fractions demonstrated potent anti-inflammatory and anti-acetycholinesterase activities
• AE and hexane fraction were found non-cytotoxic against Vero cells

Abstract

Antidesma madagascariense Lam. (AM) is used in the treatment and management of a panoply of human diseases. Leaf decoction, acetone extracts and fractions of AM were evaluated for antimicrobial, antioxidant, anti-inflammatory, cytotoxicity and acetylcholinesterase (AChE) inhibitory activity. The antibiotic-potentiating activity of crude acetone extract (AE) was assessed against ATCC bacterial strains of *E. coli* ATCC 25922, *P. aeruginosa* ATCC 27853 and *S. aureus* using standard antibiotics. Decoction extract showed significant inhibitory activity against *Acinetobacter spp.* (MIC: 0.25 mg/ml) which had higher inhibitory effect compared to the positive control Streptomycin (MIC: 1.00 mg/ml). Acetone and decoction extracts inhibited the growth of *C. albicans* (MIC: 4.00 mg/ml). All fractions of AE showed broad spectrum activity against ATCC and clinical strains (MIC 0.03- 4.00 mg/ml). The results of the combination profiles of the AE with Ciprofloxacin, Chloramphenicol and Streptomycin revealed synergistic interactions (FICI $\leq$ 0.50) against *P. aeruginosa*. Crude decoction, acetone extract and fractions were found to be potent reducing agent as well DPPH radical scavenger and ABTS cation scavenger. A significant correlation between DPPH, ABTS and total phenolic content ($p<0.05$, $r = -0.75$ and -0.82 respectively) was recorded. Three fractions namely DCM:MeOH (85:15) (F5), DCM:MeOH (95:05) (F3) and DCM (F2) [IC_{50} 0.02-0.09 mg/ml] demonstrated significant anti-inflammatory activity as compared to the positive control, diclofenac sodium [IC_{50} 0.18 (0.10-0.31) mg/ml). AE and its fractions showed AChE inhibitory activities at an IC_{50} of 35.92-492.6 µg/ml. Cytotoxicity study against Vero cells revealed that AE and hexane fraction were non-cytotoxic while decoction showed cytotoxic effect. Further studies are required to explore the potential of AM crude extracts and fractions as natural source of antimicrobial, antioxidant, anti-inflammatory agents as well as AChE inhibitors.

Keywords: *Antidesma madagascariense*; antimicrobial; antibiotic-potentiating; antioxidant; anti-inflammatory; cytotoxicity; acetylcholinesterase; Mauritius

1. Introduction

Antidesma madagascariense Lam. (AM) belongs to the Euphorbiaceae family, its genus comprises of approximately 170-200 species (Buske et al., 2002 and Mahomoodally et al., 2015). This specie also known as 'Bois bigaignon' is indigenous to the Mascarene Islands including Madagascar (Gurib-Fakim, 2007 and Mahomoodally et al., 2015). AM has been traditionally used for the treatment and management of several ailments (Gurib-Fakim, 2007 and Mahomoodally et al., 2015). The leaf and bark decoction acts as diuretic and astringent as well as effective against fever and diabetes (Gurib-Fakim, 2007 and Mahomoodally et al., 2015). The leaf decoction is also used to wash skin infections and helps to relieve muscular and rheumatic pain (Gurib-Fakim, 2007 and Mahomoodally et al., 2015). Infusion of the leaf is used against dysentery and albumin (Gurib-Fakim, 2007 and Mahomoodally et al., 2015). Leaf decoction of AM in combination with that of *Toddalia asiatica* (L.) Lam. (Rutaceae) is used to cure jaundice (Gurib-Fakim, 2007 and Mahomoodally et al., 2015).

Methanolic and aqueous extracts as well as the fractions of AM were found to scavenge DPPH, nitric oxide (NO) and superoxide anion radicals (Mahomoodally et al., 2012). These extracts and fractions also showed non-enzymatic antiglycation and anti-lipoxygenase activity (Mahomoodally et al., 2012). Narod et al., (2004) reported the antimicrobial activity of AM crude water extracts of the leaf and stem as well as their fractions against *E. coli*, *P. aeruginosa*, *S. typhimurium*, *S. aureus*, *A. niger* and *C. albicans*. The extracts were also found to exhibit contractile properties on rat illeal smooth muscles (Narod et al., 2004). Another study showed that the methanol and aqueous extracts of AM inhibited α-glucosidase enzyme with IC_{50} values 10.40±0.26 and 1.22±0.05 µg/ml respectively which were significantly lower than the positive control acarbose (IC_{50}: 5115.73 ±3.91 µg/ml) (Picot et al., 2014).

An infection may induce the process of inflammation which causes the release of biologically active mediators to attract neutrophils, leucocytes and monocytes to the infected area and these attack foreign debris and microorganisms through phacocytosis (Agyare et al., 2013). This may then lead to the production of oxygen-free radicals such as hydrogen peroxide, superoxide anion and hydroxyl anion and excess of these agents cause tissue damage in man or animal if they overwhelm the natural antioxidants of the host such as catalase, superoxide dismutase and glutathione (Agyare et al., 2013). Therefore, antioxidants hinder the activity of

free radicals and prevent damage of cells and tissues. Alzheimer's disease (AD) is considered as a fatal and chronic neurodegenerative disease that develops slowly and worsens over time (Oztaskin et al., 2015). Moreover, strong experimental evidences have demonstrated that reactive oxygen species are associated with the pathogenesis of AD, as some cellular characteristics of this disease are either causes or effects of oxidative stress theory (Ali-Shtayeh et al., 2014). This disease is linked with tangles and plaques in the brain. About 35 million people worldwide are suffering from AD, and till now there has been no effective treatment developed against this disease. AD can be managed using acetylcholinesterase inhibitors which prevent the cholinergic degradation of acetylcholine (Oztaskin et al., 2015). Therefore, the aim of this study was to investigate the antimicrobial, antioxidant, anti-acetylcholinesterase and anti-inflammatory potential of AM extracts and fractions. The cytotoxicity of the crude extracts and hexane fraction were also evaluated.

2. Materials and methods

2.1. Extraction and fractionation

Fresh leaves of *Antidesma madagascariense* were collected from Monvert Nature Park, Mauritius. The plant was identified by the Mauritius Herbarium, Agricultural Services (Barcode number: MAU 26543). The leaves were dried at 40°C in a drying cabinet for 4 to 5 days until constant mass was obtained. Dried powdered sample was extracted *via* maceration process using acetone (10:1 solvent to dry weight ratio). The extract was dried under reduced pressure using a rotatory evaporator. Acetone crude extract was fractionated *via* flash chromatography method using hexane, dichloromethane and varying ratios of dichloromethane and methanol.

2.2. Antimicrobial activity

2.2.1. Microbial strains

The antimicrobial activity of AM crude extracts and fractions was evaluated against a panel of microorganisms sourced from American Type Culture Collection (ATCC) namely: *Escherichia coli* (ATCC 25922) *Pseudomonas aeruginosa* (ATCC 27853), *Propionibacterium acnes* (ATCC 11827 and 6919), *Staphylococcus aureus* (ATCC 25923), *Salmonella typhimurium* (ATCC 14028), *Vibrio parahaemolyticus* ATCC 17802, *Candida albicans* (ATCC 10231) and clinical isolates such as *Acinetobacter spp.*, *Escherichia coli*,

Enterococcus faecalis, *Klebsiella spp.*, *Pseudomonas aeruginosa*, *Proteus mirabilis*, *Proteus spp.*, Methillin-Resistant *Staphyloccocus aureus* (MRSA) and *Staphylococcus aureus*. The clinical isolates were obtained from Victoria Hospital, Candos, Mauritius.

2.2.2. |Broth microdilution susceptibility assay

A two-fold serial microdilution technique was used to determine the minimum inhibitory concentration values for the different extracts as described by Shai et al., (2008) with some modification. A 100 µl of each extract (16 mg/ml) was serially diluted two-fold in triplicate with sterile peptone water in 96-well Microtitre plates. Fresh bacterial and fungal inoculums were prepared and adjusted to 0.5 McFarland which were further diluted 1:100 with fresh sterile peptone water broth to yield starting inoculums of approximately 10^6 CFU/ml. One hundred microliters of bacterial or fungal culture was added to each well of bacterial and fungal plates respectively. Chloramphenicol, Ciprofloxacin, Gentamicin and Streptomycin were used as positive controls for bacteria while Nystatin and Amphotericin B were used against fungus. Negative control included sterile peptone water broth. The bacterial plates were incubated at 37^0C for 24 h while fungal plates were incubated for 48 h at 25^0C. After incubation, 40 µl of iodo-nitrotetrazolium chloride (0.2 mg/ml) was added to each well and the plates were further incubated for 20 min. Bacterial and fungal growth was denoted by red coloration. The well of lowest concentration in which no pinkish red coloration was observed was considered to be the MIC. The total activity (TA ml/g) value was calculated as the total mass extracted from 1 g of plant material divided by the MIC value (mg/ml) (Shai et al., 2008).

2.2.3. Antibiotic potentiating activity

Crude acetone extract was combined with commercial antibiotics namely Ciprofloxacin (CIP), Chloramphenicol (CHL), and Streptomycin (STR) to determine any possible synergistic activity. Three different ratios of extract to antibiotic were prepared (30:70, 50:50 and 70:30) by mixing known volume of stock solution of the extract (16 mg/ml) with CIP (0.1 mg/ml), CHL (1 mg/ml) and STR (1 mg/ml) respectively as described by de Rapper et al., 2012. The assay was carried out *via* broth microdilution susceptibility method as described in section 2.2.2. A 100 µl of each extract: antibiotic (30:70, 50:50 and 70:30) combinations were serially diluted two-fold with sterile peptone water, in triplicate in a 96-well microplate for each of the ATCC bacterial strains. The different antibiotics (CIP, CHL and STR) were used alone as positive controls and sterile peptone water as negative control.

Inoculum (100 µl) was added to each well. The plates were then incubated overnight at 37 ºC. After incubation, 40 µl of INT (0.2 mg/ml) was added to each well and the plates were further incubated for 20 min at 37ºC. The MICs were recorded and the results of the combined effects of the antibiotics and extracts were calculated and expressed in terms of a fractional inhibitory concentration (FIC) index which is denoted by the following formula:

$FIC_{extract}$ = MIC of extract in combination/MIC of extract alone

$FIC_{antibiotic}$= MIC of antibiotic in combination/MIC of antibiotic alone

FIC index = $FIC_{extract}$ + $FIC_{antiobiotic,}$

$FIC_{extract}$ is the fractional inhibitory concentration of the extract and $FIC_{antiobiotic}$ is the fractional inhibitory concentration of the antibiotic used. The results were considered as a synergy if the FIC index of the combination is ≤0.5, additive when it was 0.5 <FIC index <1, subtractive when FIC index is greater than 1 and less than 4 and antagonism for FIC index > 4 (de Rapper et al., 2012, Duarte *et al.* 2012 and Chaudhary et al., 2013).

2.3. Antioxidant activity

2.3.1. Radical scavenging activity using DPPH$^{\bullet}$ method

The free radical scavenging capacity of extracts was spectrophotometrically assessed using DPPH (Hwang et al., 2001 cited Parekh *et al*. 2012). Samples (400 µg/ml, 100 µl) were serially diluted using methanol (100 µl) in a 96-well microtiter plate. Methanolic solution of DPPH (100 µM, 200 µl) was added to each well and the plate was incubated at 37 °C for 30 min. The experiments were performed in triplicate and the absorbance was measured at 517 nm. Ascorbic acid and trolox were used as positive control and methanol as the blank. The percentage inhibition was calculated using the following equation:

Percentage inhibition = $[(A_0 – A_1)/A_0]$ x 100, where A_0 is the absorbance of the blank and A_1 is the absorbance of the sample.

2.3.2. ABTS cation radical scavenging activity

The total antioxidant activity of the extracts was determined with slight modification according to Saeed et al., (2012) and Hazra et al., (2008). ABTS cation radical was generated by reacting potassium persulfate solution (15 ml, 2.45 mM) with ABTS solution (15 ml, 7 mM) and kept overnight in the dark to yield a dark blue solution. The concentration of the

resulting blue $ABTS^{\cdot+}$ was adjusted to an absorbance of 0.70 ± 0.02 at 734 nm by diluting with ethanol. Sample (400 µg/ml, 10 µl) was added to the resulting blue green ABTS cation radical solution (190 µl) in a 96 well microplate. The decrease in absorbance was measured at 734 nm for 6 min. Ascorbic acid and trolox were used as positive control and ethanol as blank. The percentage inhibition was calculated according to the formula:

Percentage inhibition = $[(A_0 – A_1)/A_0] \times 100$, where A_0 is the absorbance of the blank and A_1 is the absorbance of the sample.

2.3.3. Ferric reducing antioxidant power (FRAP) assay

The reducing capacity of the extracts was determined through FRAP assay according to a modified method of Benzie and Strain (1996). FRAP reagent was freshly prepared by mixing 10 mM of TPTZ in 40 mM hydrochloric acid, 20 mM $FeCl_3.6H_2O$ in distilled water and 300 mM acetate buffer pH 3.6. The different extracts (30 µl) were added to a 96 well plate followed by FRAP reagent (270 µl). Trolox and ascorbic acid were used as the standards. The absorbance of the Persian blue complex was measured at 593 nm after 30 min incubation at room temperature. The standard curve of Trolox was constructed and results were expressed in mM Trolox equivalent (TE) for 100 mg/L extract.

2.3.4. Total phenolic content

The total phenolic content was estimated according to the Folin-Ciocalteau's method with some modifications (Javanmardi et al., 2003). The extracts (20 µl, stock solution 400 µg/ml) and ten-fold diluted Folin-Ciocalteau phenol reagent (100 µl) were added to a 96 well plate and incubated for 5 min. Sodium carbonate solution (80 µl, 7.5%) was added to the mixture and allowed to stand for 30 min at room temperature. The resulting blue coloration was read at 760 nm. Results were expressed as mg Gallic acid equivalent (GAE)/g dry weight.

2.3.5. Determination of total flavonoid content

The total flavonoid contents (TFC) were determined using aluminium chloride colorimetric assay as reported by Tubesha et al., (2011) with slight modifications. In 96-wells plate, plant extracts (100 µl) were mixed with methanolic aluminium chloride solution (5%, 100 µl) and incubated for 10 min in the dark at room temperature. After incubation, absorbance of the resulting yellow-colored solution was measured at 405 nm. All samples were tested in triplicates and results were expressed as mg Rutin equivalent (RE)/g dry weight.

2.4. Anti-inflammatory activity

The assay was conducted using a modified method of Mahendran et al., (2015). The reaction mixture consisted of 2 mL of varying concentration of extract or positive control, diclofenac sodium, 2 mL of phosphate saline buffer (pH 6.3) and 1 mL of egg albumin. Negative control included 2 mL of phosphate saline buffer (pH 6.3) and 1 mL of egg albumin. The mixture was incubated at 37ºC for 15 min. Protein denaturation was induced by keeping the reaction mixture in a water bath at 70ºC for 10 min. The mixture was allowed to cool and turbidity was measured spectrophotometrically at 630 nm. The experiment was performed in triplicate and the percentage inhibition of protein denaturation was calculated using the following formula:

Percentage inhibition = $[(A_0 – A_1)/A_0] \times 100$, where A_0 is the absorbance of the blank and A_1 is the absorbance of the sample.

2.5. Inhibitory activity of acetylcholinesterase (AChE)

AChE inhibitory activity was determined using Ellman's colorimetric method with slight modification as described by Zheleva-Dimitrova (2013). To each well, 50 µL of extract, 140 µL of phosphate buffer pH 8, 20 µL of AChE (20 U/mL) were added and the plate was incubated for 15 min at room temperature. After incubation, 25 µL of acetylthiocholine iodide (0.71 mM) and 25 µL of 5,5'-Dithio-bis(2-nitrobenzoic) acid (DTNB, 0.5 mM) were added and the plate was further incubated for 10 min at room temperature. The absorbance was then read at 405 nm. Galanthamine was used as the positive control. The experiment was performed in triplicate and the percentage inhibition was calculated using the following formula:

% inhibition = $[(A_0 – A_1)/A_0] \times 100$, where A_0 is the absorbance of the blank and A_1 is the absorbance of the sample.

2.6. Cell culture

The Vero cell line was maintained in culture flasks containing Eagle's Minimum Essential Medium supplemented with 1% antibiotics (100 U/mL penicillin, 100 µg/mL streptomycin and 250 µg/mL fungizone) and 10% heat-inactivated fetal bovine serum, FBS). The cells were grown in a humidified incubator set at 5% CO_2 and 37◦C. After the formation of a monolayer, flask cells were sub-cultured. The cells were detached by treating them with

trypsin-EDTA (0.25% trypsin containing 0.01% EDTA) for 10 minutes followed by the addition of supplemented media to inhibit the reaction.

2.6.1. *In vitro* Cytotoxicity assay

The cytotoxicity was determined using the XTT (2, 3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide) method according to the Cell Proliferation Kit II and method described by Zheng et al. 2001. Briefly, 100 µL of Vero cells were seeded in a 96-well microtitre plate (concentration 1×10^5 cells/mL) and incubated at 37◦C and 5% CO_2 for 24 h to allow cellular attachment to the bottom of the wells. Extracts were prepared to a stock solution of 20 mg/mL, added to the microtitre plate and serially diluted to range from 400 µg/mL–1.563 µg/mL. The microtitre plate was incubated for a further 72 h. The control wells included vehicle-treated cells exposed to 2% DMSO and the positive control Actinomycin D with concentrations ranging between 0.5 μg/mL and 0.002 μg/mL. After the 72 h incubation period, 50 µL of the XTT reagent was added to a final concentration of 0.3mg/mL and the plate was then further incubated for another 2 hours. The absorbance of the colour complex was read at 490nm with a reference wavelength set at 690 nm using a BIOTEKPower-Wave XS multiwell plate reader. The assay was performed in triplicate to calculate an IC_{50} of the cell population for decoction and acetone extracts as well as hexane fraction.

2.7. Statistical analysis

All determinations were carried out in triplicates and the results were reported as mean±standard deviation. Calculation of IC_{50} was done using GraphPad Prism Version 5.03 for Windows (GraphPad Software Inc.). The data were subjected to one-way analysis of variance (ANOVA) and results with $p<0.05$ were regarded as statistically significant (Sharma et al., 2011). Correlations between antioxidant activity and total phenolic and flavonoid contents were obtained by Pearson correlation coefficient (Stagos et al., 2012).

3. Results and Discussion

3.1. Antimicrobial activity

The antimicrobial activity of crude AM acetone and aqueous extracts as well as the fractions of crude acetone were evaluated against 4 Gram positive and 9 Gram negative bacteria while *Candida albicans* was used as the candidate fungus for the evaluation of the antifungal activities of the plant. Acetone extract of AM was observed to suppress the growth of all ATCC and clinical bacterial isolate strains (MIC range: 0.25- 4.00 mg/ml). The lowest MIC

(0.25 mg/ml) was recorded against *S. aureus* ATCC strain and clinical isolates of *Enterococcus faecalis*. Noteworthy activity was observed against ATCC strains of *Salmonella typhimurium, Vibrio parahaemolyticus* and *Propionibacterium acnes* (MIC: 0.50 mg/ml). The methanol extract has been reported active against clinical isolates of *S. enteritidis* (MIC: 125 µg/ml) and *S. aureus* (MIC: 500 µg/ml) (Rangasamy et al., 2007). The reason for the good results with acetone extract could be due to the fact that acetone is a good extractant of low toxicity and high extraction capacity (Eloff, 1998, cited Eloff et al., 2011). The extract was reported to have inhibitory activity against clinical isolates of *E. coli*, *P. aeruginosa*, *E. cloacae* and *B. subtilis* at a stock concentration of 8 mg/ml *via* disk diffusion method (Rangasamy et al., 2007). The leaf decoction from the present study exhibited antimicrobial activity against all ATCC strains (MIC range: 2.00 - 4.00 mg/ml). Decoction extract displayed significant inhibitory activity against *Acinetobacter spp.* (MIC: 0.25 mg/ml) which had higher inhibitory effect compared to the positive control Streptomycin (MIC: 1.00 mg/ml). The extract showed noteworthy activity against all clinical isolates (MIC range: 0.25- 4.00 mg/ml) with the exception of *E. faecalis*. Acetone and decoction extracts were able to inhibit the growth of *C. albicans* (MIC: 4.00 mg/ml). All fractions of AM acetone extract showed broad spectrum activity against ATCC and clinical isolates (MIC range: 0.03- 4.00 mg/ml). Potency (total activity values) of the fractions was in order of F4 > F3 > F5 > F6 > F2 > F1. Fractions F3, F4, F5 and F6 inhibited the growth of ATCC strain of *S. aureus* at MIC value 0.03 mg/ml (Total antibacterial activity: 997.2-8259.0 ml/g). These fractions showed inhibitory activity against clinical isolate strain of *P. aeruginosa* at MIC value of 1.00 mg/ml which had similar effect as the positive control Streptomycin. Significant activity of these fractions were also observed against clinical isolate strains of *Klebsiella spp.* (MIC: 0.50 mg/ml). Fractions F4 and F6 revealed the highest activity against MRSA (MIC: 0.50 mg/ml) compared to the crude extracts (MIC: 4.00 mg/ml) and other fractions (MIC range: 1.00- 2.00 mg/ml). The leaves of *A. madagascariense* have been reported to contain phenols, alkaloids, cyanogenic heterosides (Gurib-Fakim and Gueho, 1996), flavonoids, cyanogenetic heterosides, leucoanthocyanins, sterols, saponins (Gurib-Fakim and Brendler, 2004, cited Mahomoodally et al., 2015), terpenes, hydrolysable tannins such as carpusin and the dimer Antidesmin (Gurib-Fakim, 2011). Thus, it could be deduced that these metabolites could be responsible for the antimicrobial activity of the extracts and fractions.

Table 1: Antimicrobial activity of crude extracts and fractions

Extracts/ Fractions	**EC[a]**	**SA[a]**	**ST[a]**	**VB[a]**	**Pa[a1]**	**Pa[a2]**	**PM[b]**	**MRSA[b]**	**EF[b]**	**PA[b]**	**SA[b]**	**EC[b]**	**AC[b]**	**KL[b]**	**PR[b]**	**CA[a]**
Decoction	2.00[x] (32.77)	2.00 (32.77)	2.00 (32.77)	2.00 (32.77)	-	-	4.00 (16.39)	4.00 (16.39)	-	2.00 (32.77)	2.00 (32.77)	4.00 (16.39)	0.25 (262.16)	1.00 (65.54)	1.00 (65.54)	4.00 (16.39)
Acetone	4.00 (18.83)	0.25 (301.20)	0.50 (150.60)	4.00 (18.83)	0.50 (150.60)	0.50 (150.60)	4.00 (18.83)	4.00 (18.83)	0.25 (301.20)	4.00 (18.83)	1.00 (75.30)	1.00 (75.30)	4.00 (18.83)	1.00 (75.30)	1.00 (75.3)	4.00 (18.83)
Hexane **(F1)**	2.00 (23.34)	2.00 (23.34)	2.00 (23.34)	-	NT	NT	4.00 (11.67)	-	4.00 (11.67)	-	4.00 (11.67)	4.00 (11.67)	-	-	-	-
DCM **(F2)**	1.00 (175.5)	1.00 (175.5)	2.00 (87.74)	4.00 (43.87)	NT	NT	4.00 (43.87)	-	-	4.00 (43.87)	4.00 (43.87)	4.00 (43.87)	4.00 (43.87)	2.00 (87.74)	-	-
DCM:MeOH (95:05) **(F3)**	0.25 (991.1)	0.03 (8259.0)	2.00 (123.9)	2.00 (123.9)	NT	NT	2.00 (123.9)	2.00 (123.9)	-	1.00 (247.8)	2.00 (123.90)	4.00 (61.94)	2.00 (123.90)	0.50 (495.50)	2.00 (123.88)	-
DCM:MeOH (90:10) **(F4)**	0.25 (725.1)	0.03 (6042.2)	0.25 (725.1)	0.50 (362.5)	NT	NT	0.50 (362.50)	0.50 (362.50)	2.00 (90.63)	1.00 (181.30)	0.50 (362.50)	1.00 (181.3)	1.00 (181.3)	0.50 (362.5)	2.00 (90.63)	4.00 (45.32)
DCM:MeOH (85:15) **(F5)**	0.25 (278.60)	0.03 (2321.40)	0.50 (139.30)	4.00 (17.41)	NT	NT	2.00 34.82)	1.00 (69.64)	-	1.00 (69.64)	2.00 (34.82)	2.00 (34.82)	2.00 (34.82)	0.50 (139.9)	2.00 (34.82)	-
DCM:MeOH (80:20) **(F6)**	0.25 (119.70)	0.03 (997.20)	0.50 (59.83)	2.00 (14.96)	NT	NT	4.00 (7.48)	0.50 (59.83)	-	1.00 (29.92)	1.00 (29.92)	2.00 (29.92)	4.00 (7.48)	0.50 (59.84)	2.00 (29.92)	-
CIP*	0.0039	0.0002	0.0008	0.0002	NT	NT	0.0008	0.001	0.0008	0.004	+	+	0.004	0.008	+	
CHL*	0.0004	0.0078	0.0031	0.0008	NT	NT	0.0031	0.008	0.0040	0.006	0.003	0.008	0.063	0.030	0.03	
STR*	0.0156	0.0039	+	+	NT	NT	+	+	0.0080	1.00	0.008	0.008	1.00	+	0.02	
TET*	NT	NT	NT	NT	0.0016	0.0008	NT	NT	NT	NT	NT	NT	NT	NT	NT	
NYS**																0.016
AM B**																0.063

F: fractions; [x]: Minimum inhibitory concentration; DCM: dichloromethane; MeOH: methanol; [a]: ATCC strains; EC*: Escherichia coli* ATCC 25922; Pa[1]: *Propionibacterium acnes* ATCC 11827; Pa[2]: *Propionibacterium acnes* ATCC 6919; PA: *Pseudomonas aeruginosa* ATCC 27853; ST: *Salmonella typhimurium* ATCC 14028; SA: *Staphylococcus aureus* ATCC 25923; VP: *Vibrio parahaemolyticus* ATCC 17802; [b]: clinical isolates; AC: *Acinetobacter spp*; EC*: Escherichia coli;* EF: *Enterococcus faecalis*; KL: *Klebsiella spp*; PR: *Proteus spp*; PM: *Proteus mirabilis*; SA: *Staphylococcus aureus;* MRSA: Methillin-Resistant *Staphyloccocus aureus*; CA: *Candida albicans* ATCC 10231; *Positive control for bacteria; **: Positive control for fungus; CHL: Chloramphenicol; CIP: Ciprofloxacin; GEN: Gentamicin; STR: Streptomycin; TET: tetracycline; AM B: Amphotericin B; NYS: Nystatin. ( ): total activity in ml/g; -: no activity; +: no bacterial growth at a stock concentration of 10 mg/ml. All data represent the mean of three independent experiments.

3.2. Antibiotic potentiating activity of crude acetone extract

Acetone crude extract (AE) was further tested in combination with commercial antibiotics such ciprofloxacin (CIP), chloramphenicol (CHL) and streptomycin (STR) at different ratios (Table 2) to assess the potentiating effect of the crude extract (Fig. 1).

Table 2: Antibiotic potentiating activity of crude acetone extract

Control (%)	**MIC (µg/mL) of Control**			**MIC (µg/mL) of AE**		
Extract (%)	**EC**	**PA**	**SA**	**EC**	**PA**	**SA**
{CIP} or [AE] alone	{0.39}	{25.00}	{0.20}	[4000]	[2000]	[250.00]
CIP (30)+AE (70)	**0.23**	**0.47**	0.23	**87.50**	**175.00**	**87.50**
CIP (50)+AE (50)	0.39	**1.78**	0.39	**62.50**	**125.00**	**62.50**
CIP (70)+ AE (30)	0.55	**1.09**	0.39	**37.50**	**75.00**	**37.50**
{CHL} or [AE] alone	{3.90}	{62.50}	{7.80}	[4000]	[2000]	[250.00]
CHL (30)+ AE (70)	**2.30**	**4.69**	**2.34**	**87.50**	**175.00**	**87.50**
CHL(50)+ AE (50)	3.90	**7.80**	**3.90**	**62.50**	**125.00**	**62.50**
CHL (70)+ AE (30)	**2.73**	**10.90**	**5.47**	**18.80**	**150.00**	**18.80**
{STR} or [AE] alone	{7.80}	{250.00}	{3.90}	[4000]	[2000]	[250.00]
STR (30)+ AE (70)	**4.69**	**4.69**	4.60	**175.00**	**175.00**	**175.00**
STR (50)+ AE (50)	7.80	**7.80**	7.80	**125.00**	**125.00**	**125.00**
STR (70)+ AE (30)	10.90	**10.90**	5.47	**75.00**	**75.00**	**37.50**

EC: *Escherichia coli* ATCC 25922; PA: *Pseudomonas aeruginosa* ATCC 27853; SA: *Staphylococcus aureus* ATCC 25923; AE: acetone extract; CIP: ciprofloxacin; CHL: chloramphenicol; STR: streptomycin; {}: MIC of control; [ ]: MIC of extract. *Bold text indicates synergistic behaviour of the antibiotic and/or extract.

Synergistic effect was observed with AE (70%) in combination of Ciprofloxacin at 30% against *E. coli*. A decrease in MIC of Ciprofloxacin was observed from 0.39 µg/ml to 0.23 µg/ml at the same combination. A remarkable decrease in MIC values of AE (from 4000 µg/ml to 37.50 µg/ml) was noted in all combinations of AE-Ciprofloxacin. All combinations of AE-ciprofloxacin exhibited synergistic behavior against *P. aeruginosa*. The MIC values of ciprofloxacin were found to decrease significantly from 25.00 to 0.47 µg/ml. The inhibitory activity of AE was noteworthy at 30% (MIC: 75.00 µg/ml). Similar trend was detected against *S. aureus*. However, the activity of ciprofloxacin was decreased in all AE-ciprofloxacin combinations which is shown by an increased in MIC value (from 0.20 to 0.39 µg/ml).

AE potentiated the activity of chloramphenicol at the concentrations of 30 and 70% which was observed by a decreased in MIC of the antibiotic (from 3.90 to 2.30 and 2.73 µg/ml respectively) when tested against *E. coli*. All AE-chloramphenicol combinations showed synergistic activity against *P. aeroginosa* and *S. aureus*.

AE (70%) enhanced the activity of streptomycin against *E. coli* which was noted by a decrease in MIC of the antibiotic (from 7.80 to 4.69 µg/ml). All AE-streptomycin combinations were found to increase the inhibitory potential of AE against *P. aeruginosa* and *S. aureus*.

Table 3: Fractional inhibitory concentration (FIC) and FIC indices (FICI) of AE in combination with conventional antibiotics

Extract [%]/ Control (%)	EC		PA		SA	
	FIC	**FICI**	**FIC**	**FICI**	**FIC**	**FICI**
AE [70]	0.02	0.61	0.09	**0.11**	0.35	1.55
CIP (30)	0.59		0.02		1.20	
AE [50]	0.02	1.02	0.06	**0.09**	0.25	2.26
CIP (50)	1.00		0.03		2.01	
AE [30]	0.15	2.96	0.04	**0.08**	0.15	2.16
CIP (70)	2.81		0.04		2.01	
AE [70]	0.02	0.61	0.09	**0.16**	0.35	0.65
CHL (30)	0.59		0.08		0.30	
AE [50]	0.02	1.02	0.06	**0.19**	0.25	0.75
CHL (50)	1.00		0.12		0.50	
AE [30]	0.004	0.70	0.09	**0.16**	0.15	0.85
CHL (70)	0.70		0.08		0.70	
AE [70]	0.04	**0.34**	0.09	**0.11**	0.70	1.90
STR (30)	0.30		0.02		1.20	
AE [50]	0.03	0.53	0.06	**0.09**	0.50	2.50
STR (50)	0.50		0.03		2.00	
AE [30]	0.02	0.72	0.04	**0.08**	0.15	1.55
STR (70)	0.70		0.04		1.40	

FIC: fractional inhibitory concentration; FICI: fractional inhibitory concentration index; AE: acetone extract; CIP: ciprofloxacin; CHL: chloramphenicol; STR: streptomycin; EC: *Escherichia coli* ATCC 25922; PA: *Pseudomonas aeruginosa* ATCC 27853; SA*: Staphylococcus aureus* ATCC 25923. *Synergistic activity is indicated with figures in bold

At low concentration of ciprofloxacin (30%), an additive interaction was detected against *E. coli* while increasing the concentration to 70% caused a shift to subtractive effect (FICI range: 0.61-2.96). When combined with chloramphenicol (30% and 70%), AE showed

additive behavior against *E. coli*. However, at AE-chloramphenicol (50:50%), *E. coli* was inhibited *via* subtraction interaction (FICI: 1.02). AE-streptomycin (70:30 %) combination revealed synergistic behavior against *E. coli* while other combinations showed an additive activity. All AE-antibiotics combinations showed synergistic activity against *P. aeruginosa* (FICI range: 0.08-0.16). All concentrations of AE combined with ciprofloxacin showed a subtractive behaviour (FICI range: 1.55 to 2.26) against *S. aureus*. Subtraction interaction was also observed for AE-streptomycin combinations against *S. aureus* (FICI range: 1.55-2.50). An additive effect was noted for all AE-chloramphenicol combinations against *S. aureus* with FICI ranging from 0.65 to 0.85.

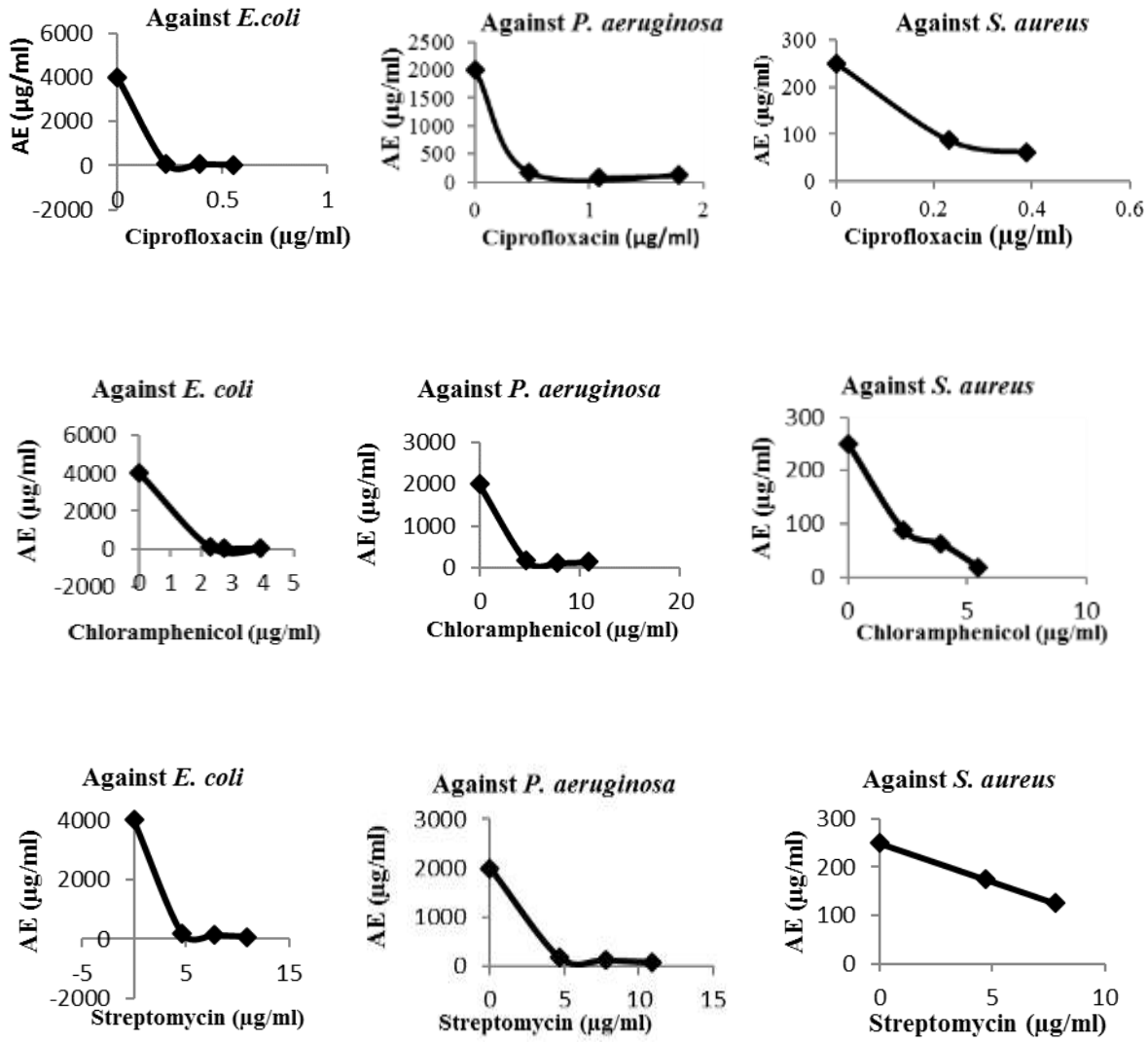

Fig. 1: Activity of AE in combination with conventional antibiotics against ATCC strains.

3.3. Antioxidant activity

Medicinal plants are widely used as alternative therapeutic tools for the prevention or treatment of many diseases (Kaur and Mondal, 2014). The generation of free radicals including reactive oxygen species namely superoxide anion radical ($O_2^{\bullet-}$), hydroxyl radical ($OH^{\bullet}$) and hydrogen peroxide (H_2O_2) during metabolism can damage macromolecules such as fatty acids, proteins and DNA which can cause several diseases including neurodegenerative disorders, cardiovascular, cancers and ageing-related disorders (Oztaskin et al., 2015). Various synthetic antioxidants, such as butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), and tertiary butyl hydroquinone (TBHQ), are used in the food industry. However, concerns have been raised about using synthetic antioxidants due to their possible side-effects, which has given impetus to finding alternative "natural" antioxidants (Kim et al., 2013). In this study, the antioxidant capacity of decoction and crude acetone (AE) extracts as well as the fractions of AE were evaluated using DPPH, ABTS and FRAP assays (Table 4). All extracts and fractions were able to reduce the stable violet DPPH radical to yellow DPPH-H. The highest antioxidant activity was demonstrated by F5 [IC_{50} (range): 1.26 (0.68-2.36) µg/ml], F4 [IC_{50} (range): 1.30 (0.70-2.42) µg/ml] and F6 [IC_{50} (range): 1.61 (0.93-2.80) µg/ml] which were significantly ($p<0.05$) lower than the positive control, ascorbic acid [IC_{50} (range): 5.89 (4.96-6.99) µg/ml]. The DPPH scavenging activities were in the order of Trolox > F5 > F4 > F6 > AE > ascorbic acid > decoction > F1 > F2. Acetone extract, F5, F4 and F6 were found to be a better ABTS radical cation scavenger [IC_{50} (range): 3.49 – 4.85 µg/ml] than the positive control ascorbic acid [IC_{50} (range): 4.91 (4.51-5.34) µg/ml]. Hexane and dichloromethane fractions were unable to quench ABTS cation radical (Percentage inhibition < 20%). The activity for the ABTS assay was in the order of Trolox > AE > F5 > F4 > F6 > ascorbic acid > F3 > decoction. The reducing capacity of a compound may serve as a significant indicator of its potential antioxidant activity (Mandal et al., 2011). FRAP assay measures the reducing potential of an antioxidant reacting with a ferric tripyridyltriazine [Fe3+-TPTZ] complex and produce an intensely blue colored ferrous tripyridyltriazine [Fe2+-TPTZ] complex in acidic medium (Mahendran et al., 2015). Generally, the reducing properties are associated with the presence of compounds which exert their action by breaking the free radical chain by donating a hydrogen atom (Nishaa et al., 2012). The reducing power of ascorbic acid (FRAP value: 869.40±43.81 µM TE) was found to be higher than all the tested extracts and fractions. However, AE (FRAP value:

323.60±18.58 µM TE) was a potent hydrogen donor as compared to decoction extract as well as its fractions (FRAP value: 38.55±2.38 - 154.66±3.68 µM TE).

3.4. Anti-inflammatory activity

The *in vitro* anti-inflammatory activity of crude extracts and AE fractions were determined against denaturation of egg albumin. Protein denaturation is a process in which proteins lose their tertiary structure and secondary structure by application of external stress or compound including strong acid or base, a concentrated inorganic salt, an organic solvent or heat (Leelaprakash and Dass, 2011). Protein denaturation can occur when there is an alteration in electrostatic, hydrogen, hydrophobic and disulphide bonding. (Mahendran et al., 2015). Biological proteins lose their biological function when denatured (Leelaprakash and Dass, 2011). F2, F3 and F5 [IC_{50} (range): 0.02-0.09 mg/ml] demonstrated significant anti-inflammatory activity as compared to the positive control, diclofenac sodium [IC_{50} (range): 0.18 (0.10-0.31) mg/ml). Decoction and F4 were unable to inhibit protein denaturation. Non-steroidal anti-inflammatory drugs are commonly used for management of inflammatory conditions but these drugs have showed some adverse effects including gastric irritation which can lead to gastric ulcers (Chandra et al., 2012). Thus, AE and its fractions represent a novel source of anti-inflammatory agent.

3.5. Acetylcholinesterase inhibitory activity

AD is controlled by extending the action of acetyl-choline (ACh) *via* acetylcholinesterase (AChE) inhibition (Machado et al., 2015). Plant-derived alkaloids namely rivastigmine and galantamine are used against AD. These drugs are AChE inhibitors and can be used to cure early and moderate stages of AD by increasing the endogenous levels of acetylcholine to boost cholinergic neurotransmission. However, these drugs have demonstrated some adverse side effects, such as hepatotoxicity and gastrointestinal disorder. Thus, there is a need to find new AChE inhibitors from natural sources, but with few side effects (Machado et al., 2015). In this study, acetone extract [IC_{50} (range): 35.97 (32.07-40.36 µg/ml) was the most potent inhibitor of AChE compared to its fractions [IC_{50} (range): 289.9-492.6 µg/ml)] but its effect was significantly lower than the positive control, galanthamine with an IC_{50} value of 3.58 (2.87-4.46) µg/ml. Decoction extract as well as fractions 1, 3 and 5 could not inhibit AChE at the highest concentration tested (2 mg/ml).

Table 4: Biological activities of crude extracts and fractions

Extract/ fractions	DPPH [IC50 (range) µg/mL]	$ABTS^{\bullet+}$ [IC50 (range) µg/mL]	FRAP (TE µM crude extract)	Anti-inflammatory [IC50 (range) mg/mL]	AChE [IC50 (range) µg/mL]	Cytotoxicity using Vero cell line[1] (IC_{50} µg/ml)
Acetone	5.22 (4.84-5.64)	3.49 (3.21-3.79)	323.60±18.58[*a]	0.99 (0.84-1.16)	35.97 (32.07-40.36)	201.85±9.28
Decoction	9.52 (7.88-11.50)	13.59 (11.10-16.64)	38.55±2.38[*a]	-	-	4.15±1.34
Hexane **(F1)**	41.28 (39.44-43.21)	-	119.13±0.22[*a]	0.20 (0.195-0.204)	-	195.3±1.93
DCM **(F2)**	43.91 (37.09-51.97)	-	122.65±2.17[*a]	0.09 (0.08-0.114)[*c]	492.6 (364.8-665.3)	NT
DCM:MeOH (95:05) **(F3)**	5.93 (4.72-7.46)	10.07 (8.78-11.55)	82.36±0.65[*a]	0.06 (0.04-0.08)[*c]	-	NT
DCM:MeOH (90:10) **(F4)**	1.30 (0.70-2.42)[*a]	3.83 (3.55-4.21)[*a]	140.72±3.90[*a]	-	289.9 (231.3-363.4)	NT
DCM:MeOH (85:15) **(F5)**	1.26 (0.68-2.36)[*a]	3.61 (3.39-3.834)[*a]	154.66±3.68[*a]	0.02 (0.01-0.04)[*c]	-	NT
DCM:MeOH (80:20) **(F6)**	1.61 (0.93-2.80)[*a]	4.85 (3.04-7.76)	136.90±3.25[*a]	0.17 (0.13-0.22)	470.0 (376.5-586.8)	NT
Control	5.89 (4.96-6.99)[a]	4.91 (4.51-5.34)[a]	869.40±43.81[a]	0.18 (0.10-0.31)[c]	3.58 (2.87-4.46)[d]	<0.05[e]
	0.96 (0.89-1.05)[b]	2.92 (2.59-3.28)[b]				

F: fractions of acetone extract; DCM: dichloromethane; MeOH: methanol; DPPH: 2,2-diphenyl-1-picrylhydrazyl radical; $ABTS^{\bullet+}$: 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid radical cation; FRAP: Ferric reducing antioxidant power; TE: trolox equivalent; AChE: acetylcholinesterase; NT: not tested; -: no activity; Control: [a]ascorbic acid; [b]trolox; [c]diclofenac; [d]galanthamine; [e]: actinomycin D; [1]: Normal adult African green monkey kidney cells. All data represent the mean ± standard deviation of three independent analyses.

IC_{50} (range) was calculated using GraphPad Prism 5.03. Difference between the samples and controls was determined using one way ANOVA with statistical significance $p<0.05$ *via* GraphPad Prism 5.03. [*]Values significantly ($p<0.05$) lower than control.

3.6. Cytotoxicity effect

Cytotoxicity testing is important to assess and validate the safety of medicinal plants for traditional use and also serve as a guide in the quest for novel active compounds (Amer, 2014). The cytotoxicity of decoction and acetone as well as the hexane fraction of AE were determined using normal adult African green monkey kidney cells (Vero cells). Acetone extract and hexane fraction were found to be non-cytotoxic with IC_{50} values 201.85±9.28 and 195.3±1.93 µg/ml. However, decoction extract was found to be cytotoxic (IC_{50}: 4.15±1.34 µg/ml) but the cytotoxic effect was lower than the positive control, Actinomycin D (IC_{50}: <0.05 µg/ml).

3.7. Total phenolic and total flavonoid contents

Phenolic compounds are of increasing interest nowadays since they delay the oxidative degradation of biomolecules (Heisanam et al., 2015). Phenolic compounds are responsible for the antioxidant activity of plants. The antioxidant capacity of medicinal plants is due to the redox properties of phenolic compounds, which enable them to act as reducing agents, hydrogen donors and singlet oxygen scavengers. Moreover, the hydrogen-donating substituents (hydroxyl groups) attached to the aromatic ring structures of flavonoids enhance a redox reaction, which in turn, help them scavenge free radicals (Adewusi et al., 2011). A high total phenolic content was observed in AE, F3, F4, F5 and F6 (GAE: 314.83±8.07 µM – 652.94±1.90 µM). However, the total flavonoid content recorded were found to be significantly low for AE and decoction extracts as well as the fractions with exception of F2 (RE range: 15.23±1.03 to 252.74±3.44 µM).

Table 5: Total phenolic and flavonoid contents of extracts and fractions

Extract/ fractions	TPC (GAE µM)	TFC (RE µM)
Acetone	363.50±4.51	18.70±1.90
Decoction	161.29±4.88	38.28±1.91
F1	81.30±4.70	15.23±1.03
F2	176.33±6.81	252.74±3.44
F3	314.83±8.07	47.42±0.72
F4	619.27±8.10	76.47±2.26
F5	665.47±2.53	87.47±2.84
F6	652.94±1.90	52.40±3.66

F: fractions from crude acetone extract; F1: hexane; F2: DCM; F3: DCM:MeOH (95:05); F4:DCM:MeOH (90:10); F5: DCM:MeOH (85:15); F6: DCM:MeOH (80:20); DCM: dichloromethane; MeOH: methanol; TPC: Total phenolic content; GAE: Gallic acid equivalent; RE: Rutin equivalent; TFC: Total flavonoid content. All data represent the mean ± standard deviation of three independent analyses.

3.8. Correlation between antioxidant activity and phytochemical content

DPPH and ABTS showed a negative relationship with total phenolic content while a positive relationship was obtained for DPPH with total flavonoid content (Table 6). The results of Pearson's correlation coefficient indicated that there was a significant correlation between DPPH, ABTS and total phenolic content ($p<0.05$, $r = -0.75$ and -0.82 respectively). A positive correlation was observed between DPPH and ABTS assay ($r = 0.86$, $p<0.05$). However, there was no correlation between antioxidant activity and total flavonoid content ($p>0.05$). The low correlation for TFC could be explained with the low content of these compounds as compared to TPC. Furthermore, a negative relationship was obtained for DPPH, ABTS and FRAPS assays.

Table 6: Pearson's correlation coefficients (r) of DPPH, ABTS, FRAP, TPF and TFC

	DPPH	ABTS	FRAP	TPC	TFC
DPPH	1.00	0.86^{*}	-0.18^{ns}	-0.75^{*}	0.48^{ns}
ABTS		1.00	-0.74^{ns}	-0.82^{*}	-0.34^{ns}
FRAP			1.00	0.26^{ns}	-0.15^{ns}
TPC				1.00	-0.08^{ns}
TFC					1.00

DPPH: 2,2-diphenyl-1-picrylhydrazyl radical; $ABTS^{\bullet+}$: 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid radical cation; FRAP: Ferric reducing antioxidant power; TPC: Total phenolic content, TFC: Total flavonoid content. Correlation was obtained using a standard Pearson correlation *via* GraphPad Prism 5.03. ^{ns}: not significant ($p>0.05$); *: significant ($p<0.05$).

4. Conclusion

The data obtained from the present study tend to validate the traditional uses of the plant against infectious diseases and inflammatory conditions. Acetone crude extract could efficiently decrease the MIC values of chloramphenicol, ciprofloxacin and streptomycin against ATCC strains of *E. coli*, *P. aeruginosa* and *S. aureus*. The synergistic effect of AE in combination with commercial antibiotics could lead to new developments for the treatment of infections caused by *E. coli*, *P. aeruginosa* and *S. aureus*. Crude decoction and acetone extract as well as its fractions were found to be potent reducing agent, DPPH radical scavenger and ABTS cation scavenger. There was a significant correlation between DPPH, ABTS and total phenolic contents. AE and its fractions have showed anti-inflammatory as well as anti-acetylcholinesterase activity. Despite the fact that AE has been found non-cytotoxic, acute toxicity studies should be conducted using appropriate *in vivo* models to assess its safety.

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