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Antioxidant potential of *Allium sativum*, *Cinnamomum zeylanicum* and *Azadirachta indica* against free radicals and their antimicrobial activity against isolated microbes from diseased Tilapia

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Abstract
Present study dealt with antioxidant activity, minerals profile and antimicrobial activity of *Azadirachta indica*, *Allium sativum* and *Cinnamomum zeylanicum* conducted in 2014. Total phenolic compound was higher in *A. indica* (279.64 ±17.34 mg GAE/100g) moderate in *A. sativum* (40.80 ± 2.91 mg) and lower in *C. zeylanicum* (11.66 ± 1.76 mg) similar in case of flavonoids content. Antioxidant activity was assessed *in vitro* using FRAP, DPPH, ABTS free radicals and copper and iron chelating activity, superoxide and hydroxyl scavenging. Results revealed plants extracts have strong antioxidant and free radical scavenging activity. In order to verify antimicrobial activity, *Pseudomonas sp.* and *Aeromonas hyrophila* were isolated from unhealthy tilapia. Ethanolic extract of *A. indica* showed maximum inhibition against both strains. *A. sativum* was moderate and *C. zeylanicum* least in action. It was concluded that test plants have strong antioxidant and antimicrobial activity and could be used in pharmaceutical formulation against various disorders.

Keywords: Antimicrobial, Antioxidant, activity, phenolic compounds, plant extract

1. Introduction
The plant material gained much interest as home remedy of various health disorders [1, 2]. Various medicinal plants including garlic, cinnamon and neem has been used extensively in the new era of pharmacy. The 70% population around the globe are using plant derived medicine against various health disorder [3]. The main cause of the health disorders is the generation of oxidative stress due to free radicals in the human body [4, 5]. The oxidative stress is generated due to various factors including UV radiations certain chemical and exposures to metals [6, 7]. The human skin receives 10^5 hits of oxidative stress on daily basis. However, the body internal antioxidant system and stability of DNA molecule lower the risk of cancer and other oxidative related diseases [7-10]. This ability deceases with aging and natural antioxidants are required in the food in order to restore the defensive mechanism against free radicals [2, 11]. The plant materials show antioxidant ability which can be linked to the presence of phenolic and flavonoid compounds [2]. Phenolic compounds exist naturally in many edible and non-edible plants in different quantity. The aromatic structure of phenolic compounds is significant for the scavenging and preventing the generation of free radicals. Flavonoid is the most important phenolic compound that play protective role against cancer and heart diseases. Additionally, they also possess antibacterial, antifungal and antiviral activity [12].
Microbial infections play a critical role in the mortality of farm fishes. The *Aeromonas* causes extensive bleeding to outside in infected fish. The blood accumulates on operculum, skin or gills. The infections of *Pseudomonas sp.* are evidenced by pale and watery appearance of internal organs and rapture in gall bladder [13]. It also causes Pseudomonas septicemia, ulcerative syndrome characterized by detached scales, darkening of the skin, petechial hemorrhage, abdominal ascites and exophthalmia [14, 15]. Uses of vaccine and antibiotics are most common method to inhibit the fish microbes. The frequent and repeated uses of vaccine and antibiotics create resistance in the pathogenic bacteria [16].

Over 90% strains of pneumococci, staphylococci and enterococci show resistance against wide range of antibiotics. Therefore, the attention was diverted towards the phenolic compounds which affect the cell membrane of the microbes [12, 17]. This study was aimed to estimate total phenolic compounds, antioxidant activity and antimicrobial activity of garlic, cinnamon and neem extract.

2. Materials and Methods

2.1 Sample collection

This study was conduct in April- October, 2014. The samples of garlic were collected from local fields located in the district Okara. The leaves of neem were also collected from the same locality. Dry cinnamon bark of analytical grade imported from Sri Lanka was purchased from scientific store and grand in the electric grinder to fine powder form. The cloves of garlic and leaves were initially crushed in grinder and air dried. The dried garlic, neem and cinnamon were grinded in the electric grinder to attain the fine powder form. The powders of all three plants were mashed through fine fabric having 100 to 120 µm mesh size. The moisture content, dry matter, total acidity and pH was determined according to the methods mentioned in the AACC [18].

2.2 Minerals content

The protocol mentioned in AOAC [19] was followed for mineral estimation. About 1 g of each sample was taken in the crucible and ignited in the muffle furnace (EHRET TK/L 4105) at 550 °C for period of 2 hours. The ash formed was dissolved in 10 ml HNO3 (10%) heated slowly for 20 min and then filtered upon cooling. Na, K was estimated through Jenway (PFP-7) flame photometer while Ca, Mg, Fe, P and Zn through Aurora TRACE (AI -1200) atomic absorption spectrophotometer. The values of each mineral was calculated through standard curve method and expressed as mg/100g sample.

2.3 Total phenolic and flavonoid content

About 100 g powder of each plant sample was placed in the Soxlet apparatus for extraction. Four types extraction solvents namely; distal water, acetone, ethanol and chloroform were used for extraction. The apparatus was run for 12 hours at 60 °C. The final extract was put in Buchi® rotatory evaporator (R-200) for removal of all extraction solvent and refrigerated at 4 C° for further uses.

Folin-ciocalteau micro method was used for estimation of total phenolic content. About 60 µl extract was taken from each plant extract and diluted with deionized water making the volume of solution 4.8 ml. then 300 µl Folin-cicalteau regent was added and allowed the mixture to stand for 10 min. finally, 900 µl Na_2CO_3 (20%) was added in the mixture and placed at 40 C for 80 min. 50µl gallic acid was used as standard and absorbance was taken at 765 nm with spectrophotometer (Hitachi u-2800). The values were expressed as mg gallic acid per gram sample and calculated with the following formula.

$$\text{Total Phenolic Compound (mg/g)} = \frac{\left[\frac{SA - CA}{Slope}\right]\left(\frac{10}{U}\right)}{(2)(1000)}$$

SA= Sample absorbance
CA= Control absorbance
Slope= Slope calculated from standard curve
(10/U)= Total volume/used volume (extract)
1000= Converting factor (µg to mg).

2.4 Flavonoid content

The protocol of Shan, *et al*. [20] was followed for flavonoid content. 0.15 ml of $NaNO_2$ (0.5 M) was mixed with 2 ml of deionized water and 0.5 ml of each sample, allowed at room temperature for 5 min. Then, 0.15 ml of $AlCl_3.6H_2O$ was added to this mixture and put in the incubator for 15 min. to this mixture; 1 ml of NaOH (1M) was added and incubated for 15 minutes. The absorbance of this mixture was taken at 415 nm comparing with 50 µl rutin and expressed as mg/g rutin equivalent.

2.5 Antioxidant activity

Benzie and Strain [21] formulated the methodology for FRAP assay. In brief, 6 ml of FRAP regent was mixed with 20 ml of each sample. This mixture was then incubated for 30 min at 37 °C. The absorbance was measured at 593 nm compared with ascorbic acid and expressed as mg Vit-C equivalent.

The protocol of Brand-Williams, *et al*. [22] with slight modification was used for DPPH free radical scavenging. In brief, 0.8 mM solution of DPPH radical was prepared using 95% ethanol. 100 µl of each sample was diluted with deionized water and ethanol maintaining the ration of 1:1 and making the volume of mixture to 5.4 ml. to this solution0.6 ml DPPH solution was added and decrease in absorbance recorded after 2 min. The decrease in absorbance was compared with standard curve of Vit-C and expressed as mg Vit-C equivalent. In the case of cinnamon and neem, torlox was used as standard of absorbance.

The method of Re, *et al*. [23] with was slightly modified and used for ABTS radical cation decolorization assay. In methodology, 7 mM solution of ABTS was reduced using 2.45 mM potassium per sulphate solution and maintained in dark for next 12-16 h. The solution of free radicals of ABTS ($ABTS^+$) formed diluted with deionized water and ethanol in 1:1 ratio till absorbance values of 0.70 at 734 nm. Then 20 µl of each sample was mixed separately in this mixture and absorbance was compared with 0.20 µg ascorbic acid. The decrease of absorbance was ABTS value expressed as mg Vit-C equivalent.

$$\text{Value of FRAP, ABTS, DPPH (mg/g)} = \frac{\left[\frac{SA - CA}{Slope}\right]\left(\frac{10}{U}\right)}{(2)(1000)}$$

The results were multiply with 100 for conversion into of mg per 100g.

The copper chelating activity was measured using protocol of Sánchez-Vioque, *et al*. [24] and expressed in mg/g sample. Decker and Welch [25] method was followed for iron chelating potential. The results were expressed as mg/g EDTA due to EDTA as standard material for comparison. The methodology of Su, *et al*. [26] was followed for superoxide scavenging and expressed as mg TE equivalent/g. Zhang, *et al*. [27] was followed for hydroxyl radical scavenging and expressed in term of mg TE equivalent/g sample.

2.6 Antimicrobial activity

Two bacterial strains namely *Aeromonas hyrophila* and *Pseudomonas sp.* were isolated from unhealthy tilapia captured from head Sulemanki Punjab, Pakistan. Both strains were cultured on Brain Heart infusion Broth (BHI-DIFCO®) media and incubated at 30 °C for 24 h. The isolated microbes were confirmed using Gram staining biochemical test. After identification the microorganisms were maintained on nutrient agar slant at 4 °C.

Disc diffusion technique was used for antimicrobial activity following Newall, *et al*. [28]. The nutrient agar medium was

first sterilized at 121 °C and 15 ibs pressure for 30 min. The medium was cooled at 60 °C and poured 25 ml to petri-dish keep at room temperature to solidify and then incubated at 4 °C for further uses. The isolated bacterial strains were spread over medium using sterile spreader. About 7 mm holes were made in each dish using sterile cork borer. 50 µl from each plant extract was placed in each hole. The butylated hydroxytoluene was used as control. The experiment was repeated for each solvent extraction in order to see the effect of solvent on the antimicrobial activity of each plant extract. The saline water was used as control of the antimicrobial activity. All the plates were placed in incubators for 24 hours at 37 °C.

2.7 Statistical analysis

All samples were in three replicates and represents in results as Mean ± SD. It was compared through to key test using IBM statics (V.20). The standard curve was drawn using MS excel (V.2010) program.

3. Results

The proximate analysis showed that the garlic was rich in moisture contents having 64.20 ± 3.90 % of moisture contents. The cinnamon has less moisture due to dry bark. The total acidity was greater in garlic than neem and cinnamon (Table-1). Neem extract shows greater quantity of minerals with higher amount of calcium (1387.23 ± 317.77 mg/100g), sodium (1255.78 ± 210.12 mg/100g) and potassium (115.45 ± 200.42 mg/100g). The manganese was found in least amount (Table-2). Iron was found significantly greater amount in cinnamon then neem and less in garlic. Cinnamon also shows rich profile of minerals after neem. Similarly, total phenolic compounds were much higher in neem (279.64 ± 17.34 mg/100g) than garlic (40.80 ± 2.91 mg/100g) and cinnamon (11.55 ± 3.49 mg/100g). Neem is also rich in flavonoid content (14.55 ± 3.49 mg/100g) slightly greater than cinnamon (11.55 ± 2.37 mg/100g) and garlic (Table 3).

Table 1: Proximate analysis of raw garlic, neem and cinnamon

Plant species	Moisture	Dry matter	pH	Total acidity
A. sativum	64.20 ± 3.90^{A}	34.91 ± 8.26^{C}	5.06 ± 0.51^{A}	0.50 ± 0.089^{A}
C. zeylanicum	10.82 ± 2.21^{C}	87.66 ± 5.11^{A}	5.95 ± 1.12^{A}	0.27 ± 0.074^{B}
A. indica	46.12 ± 5.61^{B}	53.88 ± 7.01^{B}	5.42 ± 0.82^{A}	0.37 ± 0.021^{AB}

Values are mean ±SD of five replicates

Values in the same column with same letter are not significantly different at 5% probability

Table 2: Elemental analysis of Garlic, cinnamon and neem in term of mg 100 g^{-1}

Elements	*A. sativum*	*C. zeylanicum*	*A. indica*
Na	4.54±0.61	25.23±3.23	1255.78±210.12
Ca	24.79±2.78	673.54±22.35	1387.23±317.77
Fe	3.79±0.80	9.60±1.61	4.25±1.04
P	8.23±2.03	60.44±4.56	47.44±12.51
K	48.75±3.69	381.15±12.15	1115.45±200.42
Zn	0.47±0.07	0.33±0.07	4.16±0.23
Mn	0.014±0.006	0.68±0.1	0.32±0.12

Values are mean ±SD of five replicates

Values in the same row with same letter are not significantly different at 5% probability level

Table 3: Total phenolic and flavonoids content

Plant Species	Total phenolic mg GAE/100 g	flavonoids mg RE/100 g
A. sativum	40.80 ± 2.91^{B}	4.59 ± 1.28^{C}
C. zeylanicum	11.66 ± 1.76^{C}	11.55 ± 2.37^{B}
A. indica	279.64 ± 17.34^{A}	14.55 ± 3.49^{A}

Values are mean ±SD of five replicates

Values in same column with same letter are not significantly different at 5% probability level

Cinnamon showed greater copper chelating potential than garlic and neem. Similar values were in the case of iron chelating activity. However, neem shows greater superoxide scavenging along hydroxyl scavenging potential (Table 5).

Table 4: Antioxidant capacity of garlic and cinnamon extracts

Plant species	FRAP (mg/100g)	DPPH (mg/100g)	ABTS (mg/100g)
Control	23.32 ± 2.63^{D}	14.12 ± 6.64^{D}	132.24 ± 17.05^{D}
A. sativum Vit.C Equivalent	35.22 ± 6.63^{C}	28.82 ± 11.61^{C}	231.64 ± 25.02^{C}
C.zeylanicum trolox Equivalent	357.13 ± 50.90^{B}	414.91 ± 97.63^{A}	917.22 ± 125.23^{A}
A. indica trolox Equivalent	471.19 ± 59.30^{A}	214.56 ± 37.13^{B}	629.42 ± 29.56^{B}

Control: Vitamin C

Values are mean ±SD of five replicates

Values in same column with same letter are not significantly different at 5% probability level

Table 5: Comparison of free radicals scavenging ability of *A. sativum C. zeylanicum* and *A. indica*

Free radicals	*Control*	*A. sativum*	*C. zeylanicum*	*A. indica*
Copper chelating activity (mg/g)	7.14 ±0.69^{A}	21.44 ±1.19^{B}	31.66±4.75^{A}	14.62±3.45^{C}
Iron chelating activity (mg/g)	0.79± 0.09^{D}	0.69± 0.09^{C}	2.11±0.94^{A}	0.76±0.34^{B}
Superoxide scavenging (mg/g)	2.27± 0.88^{C}	4.87± 0.95^{B}	4.33±1.23^{C}	6.78±2.33^{A}
Hydroxyl scavenging (mg/g)	3.29± 0.24^{B}	9.09± 1.71^{B}	6.45±1.29^{C}	10.38±3.19^{A}

Control: Vitamin C
Values are mean ±SD of five replicates
Values in the same row with same letter are not significantly different at 5% probability level

In antimicrobial activity, *Pseudomonas sp*. was found most susceptible to all plant extracts than *A. hyrophila*. The *A. indica* was most effective in inhibiting the growth of *Pseudomonas sp. A. sativum* intermediary and C. *zeylanicum* was least in action. The *A. indica* was also effective against *A. hyrophila* (Table-6). In 2nd phase, the effect of extraction solvent on antimicrobial activity of each extract was also tested. It was found that ethanolic extract of *A. indica* was most effective as antimicrobial agent against both bacterial strains. The order of antimicrobial activity of solvent was ethanol> acetone> chloroforms (Fig. 1, 2 & 3).

Table 6: Comparison of zone of inhibition (mm) *A. sativum, C. zeylanicum A. indica* and against *Aeromonas hyrophila* and *Pseudomonas sp.* after 48 hours of incubation

Extract	Zone of Inhibition (mm)	
	Aeromonas hyrophila	*Pseudomonas sp.*
Control	6.60±1.28^{D}	4.27±1.88^{D}
A. sativum	10.29±2.25^{B}	15.27±2.12^{B}
C. zeylanicum	12.55±3.22^{B}	11.13±3.23^{C}
A. indica	20.56±5.23^{A}	27.25±5.78^{A}

Control: Butylated hydroxytoluene
Values are mean ±SD of five replicates
Values in same column with same letter are not significantly different at 5% probability level

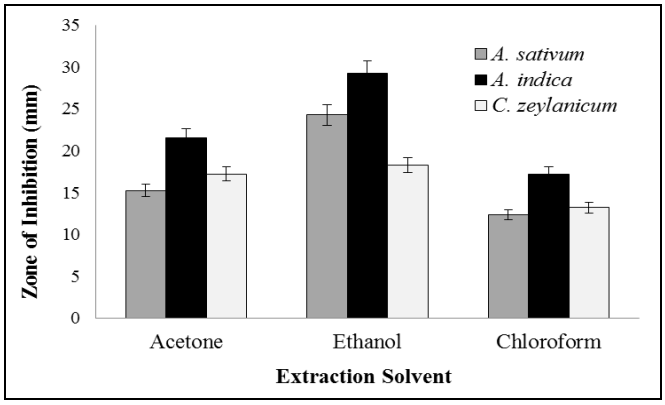


Fig 1: Effect of different extraction solvent on the antimicrobial activity of tested plants against *Pseudomonas sp*

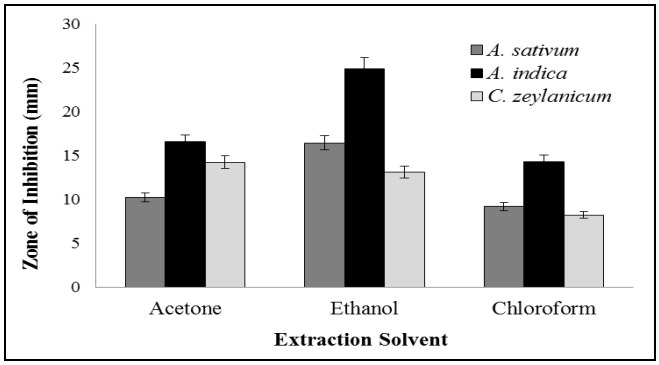
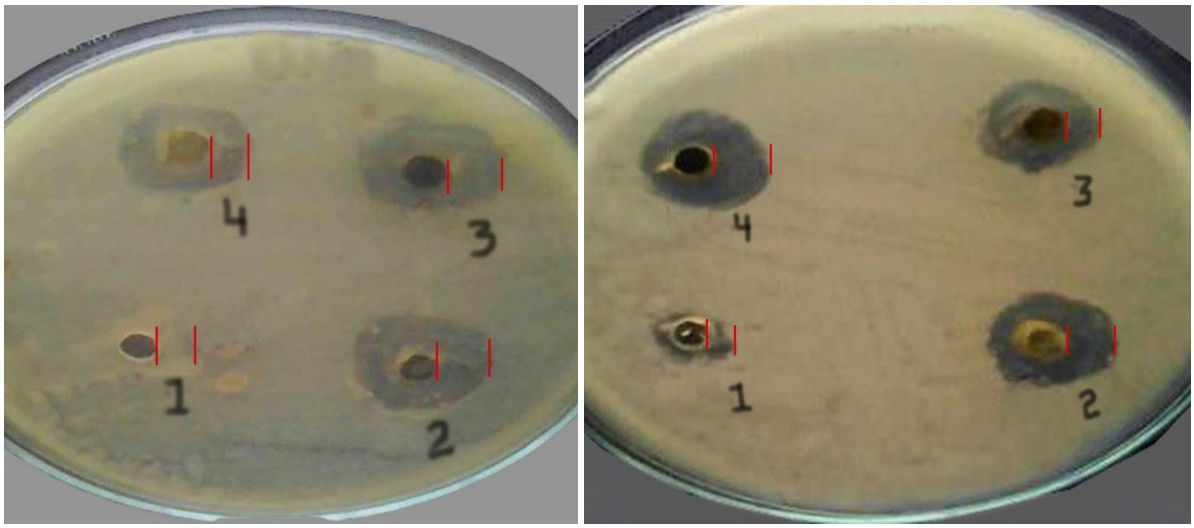


Fig 2: Effect of different extraction solvent on the antimicrobial activity of tested plants against *A. hyrophila*



A. B.

Fig 3: Zone of inhibition in *A. hyrophila* (A) and *Pseudomonas sp.* (B) culture Where, 1 is Butylated hydroxytoluene, 2 *A. indica*, 3 *C. zeylanicum* 4, *A. sativum* in both cultures

4. Discussion

Plant based medicine gain much popularity in developing countries due to fewer side effects and ease in availability [2]. Researchers are focusing on medicinal aspects of plant and explore their contents that are beneficial in cure of various health disorders [29]. Chemical composition analysis plays a

vital role to assess the nutritional quality and quantity of plant materials constituting the human diet. The garlic had 64.20 ± 3.90 % moisture analogue to studies of Khalid, *et al*. [1], neem 46.12 ± 5.61 % where the cinnamon showed 10.82 ± 2.27 % moisture similar to results of Dhillon and Amarjeet [30] how calculated 10.43 % moisture in their studies. The dry content was slightly different from the findings of Khalid, *et al*. [1] how estimated 32.73 ± 0.84 % moisture content in garlic. The difference is probably due to environmental conditions and different storage methods. The pH was acidic and non-significant difference recorded in study.

The phytochemical screening of garlic, cinnamon and neem shows presence of phenolic and flavonoids compounds which possess antioxidant activities. These compounds attain the ability due to presence of hydroxyl group in structure scavenging the endogenous free radicals [31]. The phenolic are secondary metabolites that were present higher in neem (279.64 ± 17.34) intermediate in garlic (40.80 ± 2.91) and lesser in cinnamon (11.66 ± 1.76 GAE/100g) in study. The flavonoids were also higher in neem than garlic and cinnamon (Table-3). The presence of total phenolic and flavonoids in all tested plant extracts showed excellent reducing power against FRAP, DPPH and ABTS radicals.

In DPPH reducing assay, cinnamon extract showed high affinity towards DPPH radical scavenging by donating the proton in order to neutralize free radicals generated in body. Further, the phenolic compounds formed α bond with metals ions to oxidize metal ions. The results also demonstrated that all plant extracts have capabilities to reduce the FRAP radicals which further confirmed antioxidant role of garlic, cinnamon and neem. The FRAP assay may determine the electron transfer ability of test compound in acidic environment [32]. The active compound donates the electron and reducing the Fe^{3+} to Fe^{2+}. Unexpectedly, cinnamons contained more FRAP reducing ability than garlic and neem (Table 5). This may due to different phenolic compounds present in plant species [33]. The cinnamon further showed significant ABTS radicals scavenging at pH similar to human body. The phenolic compounds probably involved in antioxidant activity against ABTS free radicals and even cooking doesn't alter or slightly affected antioxidant profile [34]. Neem showed intermediate scavenging and garlic has less scavenging ability of ABTS free radical. Siripongvutikorn [35] recorded slightly higher values for garlic and cinnamon and difference could be due to difference in extraction solvent and analytical methods. Khalid, *et al*. [1] also calculated slightly different values for *A. sativum* again possibly due to extraction solvent and analytical methods.

The plant extracts are highly desirable for prevention of pathogenic microbe's infections [36]. The plant base antimicrobial agents are extensively used due to environmental friendly nature, low extraction cost, very effective and less harmful compared to synthetic antibiotics [37]. Previously, Rattanachaikunsopon and Phumkhachorn [38] uses *Solanum trilobalum* and Christybapita, *et al*. [39] *Eclipta alba* to control fish pathogenic bacteria. Babuselvam, *et al*. [40] used extracts of *Rhizophora mucronata* and *Salichornia brachiata* against pathogenic bacteria isolated from shrimps and fishes. They found that the *Salichornia brachiata* showed high potency of antibacterial than the *Rhizophora mucronata*, against the pathogens of shrimp (*Vibrio alginolyticus*) and fish (*Vibrio parahaemolyticus*). The previous studies also showed that various secondary metabolites such as flavonoids, alkaloids, glycosides and phenolic compounds isolated from plants have strong antimicrobial activity against gram positive and gram negative bacteria [41, 42]. Therefore, the presence of phenolic and flavonoids make the neem, garlic and cinnamon as suitable antimicrobial agent against both bacterial strains.

In this study, aqueous extract of *A. indica* showed maximum inhibition against *Pseudonomos sp*. (27.25 ± 5.76 mm) and *A. hyrophila* (20.56 ± 5.23 mm). The *A. sativum* and *C. zeylanicum* were moderate in action (Table-6). In later part of study, the effect of extraction solvent on antimicrobial activity was determined. It revealed that the ethanolic extract of *A. indica* was efficient in inhibiting the growth of both bacterial strains. Further, the ethanolic extract of all plant extract show maximum inhibition against both bacterial strains (Fig. 1, 2 & 3).

5. Conclusion

In conclusion, leaves extract of *A. indica* showed maximum inhibition against *Pseudomonos sp.* and *A. hyrophila* due to higher amount of phenolic and flavonoids contents. This plant extract also showed maximum scavenging of free radicals tested in study. The *A. sativum* and *C. zeylanicum* were moderate in action. The results of this study revealed that three tested plants are medically important and can be used in future pharmaceutical formulations as antioxidant and antibacterial agent.

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