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Original Article

Antioxidant, Antibacterial and Brine Shrimp Lethality Bioassay of *Amoora cucullata*, a Mangrove Plant

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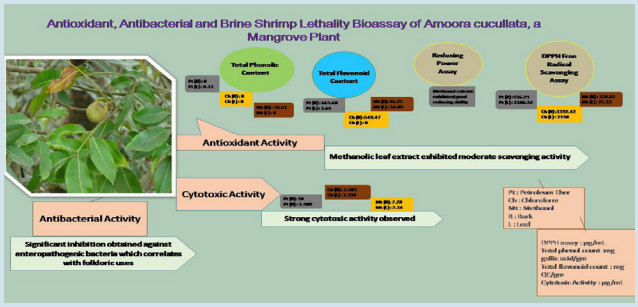
ABSTRACT

Objective: To determine antioxidant, antibacterial and brine shrimp lethality bioassay of *Amoora cucullata* using bark and leaf extracts of petroleum ether, chloroform and methanol. **Methods:** Bark and leaf parts were separately soaked into petroleum ether, chloroform and methanol respectively. To determine the presence of antioxidant activity; DPPH free radical scavenging assay, total phenolics and flavonoids determination and reducing power assay were carried out. Disk diffusion and brine shrimp lethality bioassay were conducted for the preliminary screening of antibacterial activity and cytotoxicity. **Results:** IC_{50} values in DPPH free radical scavenging assay for the extracts of petroleum ether, chloroform and methanol were found to be 316.23, 1192.42 and 128.82 in bark and 1106.32, 1330 and 25.12 µg/mL in leaf extracts respectively. Total phenolic content determined by Folin-Ciocalteu method in bark was 58.41mg gallic acid/gm of dry plant material in methanol while 8.11 mg in petroleum ether leaf extract respectively. Total flavonoid content in bark and leaf was found to be 663.60, 549.47 and 46.25 mg in petroleum ether, chloroform and methanol while 1.64 and 14.09 mg QE/gm of dried plant material in petroleum ether and methanol respectively. In reducing power assay, methanolic extracts exhibited good reducing capacity. In antibacterial activity, extracts showed significant inhibition against enteropathogenic bacteria. Besides in brine shrimp lethality bioassay LC_{50} value was found to be 10, 2.301 and 7.28 in bark and 1.308, 1.94 and 2.14 µg/mL in leaf extracts of petroleum ether, chloroform and methanol respectively. **Conclusion:** From the present research work it can be concluded that *A. cucullata* leaf possess moderate antioxidant properties; strong antibacterial activity and potential cytotoxic properties.

Key words: Antioxidant, Antibacterial, Cytotoxicity, *Amoora cucullata*.

PICTORIAL ABSTRACT



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INTRODUCTION

As means of primary healthcare, more than 80% of world population relies mainly on plants and their extracts.[1,2] However, the health care system of the remaining 20% of the population also depends on plant products, mainly in developed countries.[3] Where these plants constitute Ayurvedic, Unani, and others forms of traditional medicinal system.[4] Folk medicinal practitioners known as Kavirajes rely almost exclusively on these medicinal plants or their parts as their medicament to treat patients for various ailments.[5]

Amoora cucullata (*Meliaceae*), a rare and endangered tree species of the Sundarbans east is distributed in the rain forest of South East Asia, Burma, India, Indonesia, Malaysia, Nepal, Pakistan, Papua New Guinea, Solomon Islands, Thailand, Vietnam, Bangladesh[6,7] and called bekak, garotai, latmi but most commonly *Amoora* across the world.[7] This plant has been used as a folk medicine for treatment of marrow and diarrhea.[8] The leaves are traditionally used in the treatment of inflammation, skin diseases and in cardiac disease.[9] The crude methanolic extracts of leaves were reported to isolate polyphenols and tannins[10] and was found to show antiinflammatory, antinociceptive, diuretic and central nervous system (CNS) depressant activities.[11] The present study was carried out to determine antioxidant, antibacterial and preliminary cytotoxic activities and their rational usage in traditional medicine.

MATERIALS AND METHODS

Drugs and Chemicals

The solvents and chemicals used in the present investigation were Merck analytical grade (Germany) unless otherwise stated.

Collection of Plant Material

Different plant parts (i.e. bark and leaf) of *A. cucullata* were collected from Dhangmaree, Chadpai range of the Sundarbans East zone on 16th December, 2011 and were taxonomically identified by an experienced taxonomist at the Forestry and Wood Technology Discipline, Khulna University, Bangladesh. A voucher specimen was also deposited for future reference (F018).

Preparation and Extraction of Plant Material

Followed by careful cleaning leaves and barks were separated from each other, cut into small pieces; air dried for several weeks; ground into coarse powder, weighed (bark 122.95 gm, leaf 78 gm) and separately soaked into 500 mL and 400 mL petroleum ether respectively. They were then sealed; kept for a period of 5 days accompanying occasional shaking and stirring and then filtered after the period.

Similarly approx. 119 gm powder from barks and 75.60 gm powder from leaves of *A. cucullata* were soaked into 550 and 350 mL chloroform respectively, kept for a period of 6 days and then filtered. About 110.52 and 72 gm powder from barks and from leaves of *A. cucullata* were

soaked into 500 and 300 mL methanol respectively, kept for a period of 6 days and then filtered.

Determination of Antioxidant Activity

In this present investigation, the petroleum ether, choloroformic and methanolic bark and leaf extracts were subjected to a number of assay systems with a view to determining the presence of antioxidants in these extracts. These included the determination of free radical scavenging activity by DPPH free radical scavenging assay, determination of total phenolics and flavonoids and reducing power.

DPPH Free Radical Scavenging Activity

In determining DPPH free radical scavenging activity, different concentrations of the extracts were prepared in the rage from 1.57 to 400 µg/mL and then 2 mL of 0.004% DPPH solution was added in test tube of different extracts. The test tubes were allowed to stand at dark for 30 min to complete the reaction and then absorbance was recorded at 517nm.[12] The decrease in absorbance with blank was also measured. The experiment was carried out in triplicate.

Determination of Total Phenolic and Flavonoid Content

Total phenolic content of the extracts was determined by using Folin-Ciocalteau assay[26] where extract or standard solution (25 to 250 µg/mL) of 1 mL was added to distilled water (9 mL), and then 1 mL of FC reagent (10 times diluted with distilled water). After 5 minutes; 10 mL 7% Na_2CO_3 was added to the mixture, kept for 30 minutes and then absorbance was measured at 750 nm using UV spectrophotometer. The percentage of total phenolics was calculated from the calibration curve of gallic acid plotted by using the similar procedure as the extracts and expressed as mg gallic acid equivalent (GAE)/gm dried plant material.

Total flavonoid content of the extracts was determined by using an aluminium chloride colorometric assay[26] where extract or standard solution (25 to 400 µg/mL) of 1mL was added to distilled water (5 mL); 0.3 mL 5% $NaNO_2$ then added to the mixture followed by addition of 0.6 mL 10% $AlCl_3$ and 2 mL 1M NaOH after 5min. Then absorbance was measured at 510 nm; percentage of total flavonoids was calculated from the calibration curve of quercetin plotted by using the similar procedure as the extracts and expressed as mg quercetin equivalent (QE)/gm dried plant material.

Determination of Reducing Power

The reducing power of the extract was evaluated according to the method of Oyaizu.[13] According to this method different concentration of extracts were prepared in the range from 25 to 400 µg/mL. Then equal amount (2.5 mL) of phosphate buffer and potassium ferricyanide was mixed with 1 mL of test extract. After incubation, 2.5 mL of 10% trichloroacetic acid was added and then centrifuged. Absorbance was taken at 700 nm following addition of 0.5 mL ferric chloride.

Antibacterial Screening

Antibacterial activity of extracts was tested by disc diffusion method.[14] Seven bacterial strains (1 gram-positive and 6 gram-negative) were maintained on the nutrient agar medium (Merck, Germany). The sterile filter paper discs were prepared by adding desired concentration (500 & 1000 µg/disc) of extracts on the disc with the help of a micropipette. Standard ciprofloxacin disc (5 µg/disc), discs containing extracts and control discs were then impregnated, incubated overnight at 37°C, checked for the zone of inhibitions and then diameters of inhibition zone were measured in millimeters (mm).

Brine Shrimp Lethality Bioassay

The eggs of the brine shrimp, Artemia salina, and sea water were collected from BRAC prawn hatchery, Sreeghat, Bagerhat, Bangladesh. For hatching, it took 24 h to mature and the shrimps were then called nauplii. For the experiment, extracts were dissolved in DMSO and was added in test tubes in such a way that each contain 4 mL of sea water with different concentrations of extracts ranging from 5 to 320 µg/mL. The final volume for each test tube was adjusted to 10 mL with artificial sea water and 10 living nauplii were introduced to each tube. After observing test tubes 24 h later, the survived nauplii were counted.[15] The percentage of dead nauplii in the test and standard group was established by linear correlation when logarithm concentration versus percentage of mortality was plotted and LC_{50} value was calculated using Origin Pro.

RESULT

In the present study, antioxidant activity of petroleum ether, chloroform and methanolic bark and leaf extracts were subjected to DPPH free radical scavenging assay, assay for total phenolic and flavonoid content determination, total reducing power assay as indicators for the presence of antioxidants, disc diffusion assay for determining antibacterial activity and brine shrimp lethality bioassay for preliminary indication of cytotoxic compounds in *A. cucullata*.

DPPH Free Radical Scavenging Activity

The DPPH radicals were used for evaluating antioxidant activity because of its outstanding ability to become a stable molecule by accepting an electron.[16] The reduction of DPPH radical was determined by decrease of absorbance at 517 nm facilitated the presence of antioxidants in the extracts. Figure 1a and 1b shows the dose-dependent curve of DPPH radical activity of petroleum ether, chloroform and methanolic bark and leaf extracts of *A. cucullata* respectively. The bark extracts of petroleum ether, chloroform and methanolic solvent system exhibited 50% inhibition (IC_{50}) at a concentration of 316.23, 1192.42 and 128.82 µg/mL while the values for leaf extracts in the solvent systems were found to be 1106.32, 1330 and 25.12 µg/mL respectively (Table 1).

Determination of Total Phenol and Flavonoid Contents

The amount of total phenolic content determined in relation to gallic acid equivalent (Figure 2a) in bark was found to be 58.41 mg in methanol while in leaf 8.11 mg GAE/gm of dried plant material in petroleum ether respectively (Table 1). On the other hand, the amount of total flavonoid was determined using the calibration curve of quercetin equivalent (Figure 2b). The values were found to be 663.60, 549.47 and 46.25 mg in the bark of petroleum ether, chloroform and methanolic solvent system; while 1.64 and 14.09 mg of QE/gm of dried plant material in the leaf of petroleum ether and methanolic solvent system respectively (Table 1).

Determination of Reducing Capacity

While comparing reducing ability of extracts with highest value of standard obtained at maximum concentration (Table 1), moderate activity was found in methanolic extracts.

Antibacterial Screening

In *A. cucullata*, 1000 µg/disc of petroleum ether, chloroform and methanolic bark and leaf extracts produced maximum zone of inhibition against bacteria that causes stomach disorder and skin diseases (Table 2). It was reported that ethyl acetate and methanol bark extracts of *A. cucullata* showed moderate to strong antimicrobial activity by producing the zone of inhibition in the range of 8-14 mm and 9-16 mm, respectively.[25]

Brine Shrimp Lethality Bioassay

In brine shrimp lethality bioassay, 50% mortality rate (LC_{50}) of brine shrimp was found to be 10, 2.301 and 7.28 µg/mL in bark (Figure 3a) and 1.308, 1.98 and 2.14 µg/mL in leaf (Figure 3b) of petroleum ether, chloroform and methanolic extracts respectively (Table 3). In compared to standard vincristine sulfate (LC_{50} 0.128 µg/mL); outstanding mortality

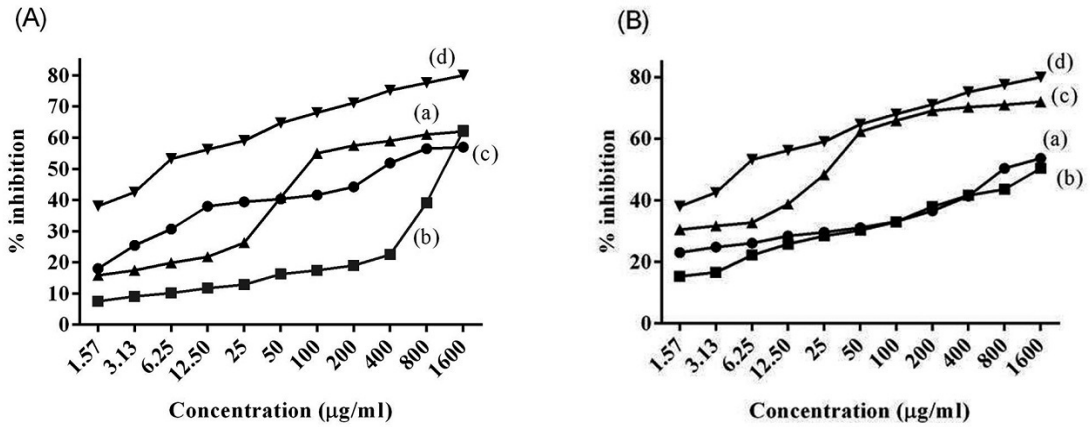

Figure 1: DPPH free radical scavenging activity of petroleum ether (a), chloroform (b), and methanolic (c), extract of *A. cucullata* bark (A), and leaf (B) in compaision with standard quercetin (d).

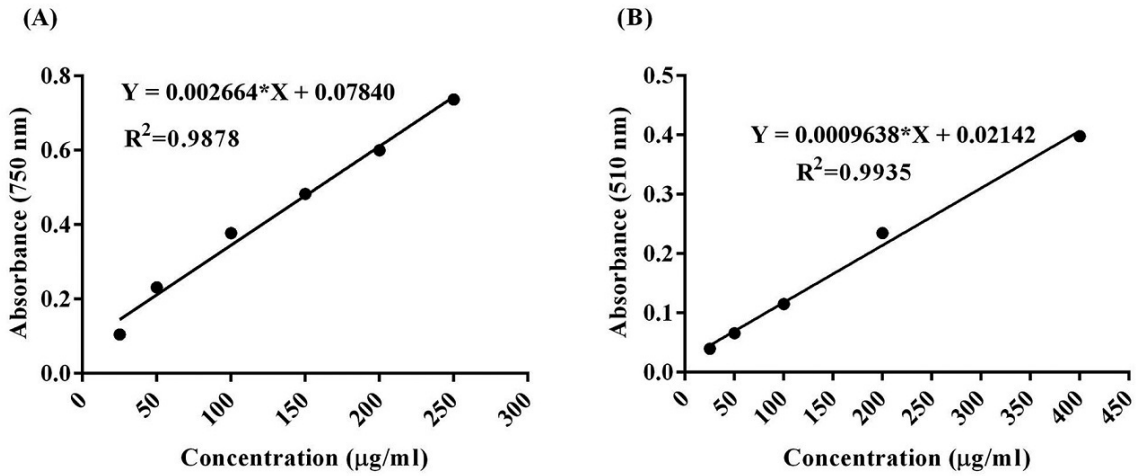

Figure 2: Standard curve for gallic acid (A) and quercetin (B)

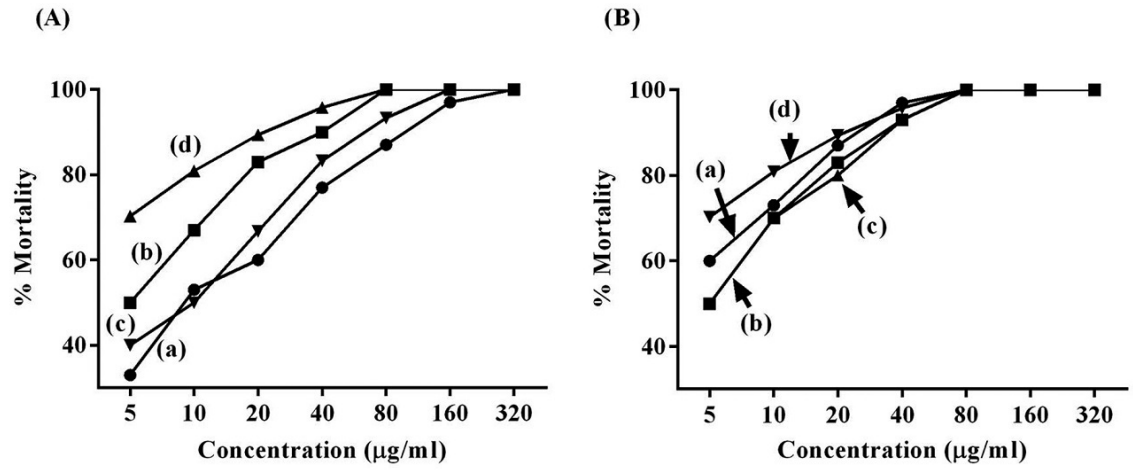

Figure 3: Brine shrimp lethality bioassay of petroleum ether (a), chloroform (b), and methanolic (c), extract of *A. cucullata* bark (A), and leaf (B) in compaision with standard vincristine sulfate

Table 1: Evaluation of antioxidant activity for petroleum ether (Pt), chloroformic (Ch) and Methanolic (Mt) barks and leaf extracts of *A. cucullata*

Name of the tests	Plant parts	Solvent systems	Values in respective units	Regression equation	R^2value
DPPH (IC_{50} value in µg/ml)	bark	Pt	316.23	y = 19.93 + 11.96x	0.908
		Ch	1192.42	y = 11.29 + 0.032x	0.979
		Mt	128.82	y = 5.29 + 21.16x	0.899
	leaf	Pt	1106.32	y = 28.98 + 0.019x	0.810
		Ch	1330	y = 26.06 + 0.018x	0.620
		Mt	25.12	y = 22.02 + 19.99x	0.928
	Standard(Quercetin)		6.64	y = 37.62 + 15.06x	0.967
Total phenol content(mg gallic acid/gm of dry plant material)	bark	Pt	-	Gallic acid calibration curve: y = 0.002664x + 0.0784	0.9878
		Ch	-		
		Mt	58.41 ± 0.008		
	leaf	Pt	8.11 ± 0.003		
		Ch	-		
		Mt	-		
Total flavonoid content (mg Quercetin/gm of dry plant material)	bark	Pt	663.60 ± 0.18	Quercetin calibration curve: y = 0.0009638x + 0.02142	0.9935
		Ch	549.47 ± 0.18		
		Mt	46.25 ± 0.01		
	leaf	Pt	1.64 ± 0.18		
		Ch	-		
		Mt	14.09 ± 0.0003		
Reducing power (highest value at maximum conc.)	bark	Pt	0.671 ± 0.007	Standard Quercetin : y=1.586+0.045x	
		Ch	0.654 ± 0.006		
		Mt	0.981 ± 0.054		
	leaf	Pt	0.594 ± 0.003		
		Ch	0.621 ± 0.005		
		Mt	0.732 ± 0.005		

Table 2: Antibacterial activity of petroleum ether (Pt), chloroform (Ch) and methanol (Mt) extract of *A. cucullata* bark, leaf and standard antibiotic ciprofloxacin as control.

	Zone of Inhibition in mm						
Bacterial Species	bark (1000 µg/disc) Mean ± SD; (n = 3)			leaf (1000 µg/disc) Mean ± SD; (n = 3)			Ciprofloxacin (5 µg/disc) Mean ± SD (n = 3)
Gram positive	pt	ch	mt	pt	ch	mt	Highest inhibition zone
Micrococcus	15.3 ± 1.2	13.7 ± 1.2	16 ± 0.82	18.7 ± 1.2	14.3 ± 1.2	13 ± 0.82	36 ± 2.8
Gram negative							
S. dysenteriae	15.3 ± 1.7	20.3 ± 1.2	11.7 ± 0.47	16.7 ± 1.2	13.7 ± 1.2	17.7 ± 1.2	37.7 ± 2.1
S. flexneri	14.3 ± 1.2	11.3 ± 1.2	15 ± 2.2	17 ± 0.82	20.7 ± 1.2	16.3 ± 1.2	35.7 ± 1.7
S. typhi	17.3 ± 0.94	17 ± 2.2	19 ± 1.2	24.3 ± 0.94	15.3 ± 0.47	12.3 ± 1.2	36.7 ± 2.1
E. coli	13.7 ± 2.06	20.3 ± 2.1	23.3 ± 1.2	22.3 ± 0.94	15 ± 1.7	15.3 ± 2.5	34.7 ± 0.94
P. aeruginosa	20.7 ± 1.7	16 ± 1.4	22 ± 1.2	21.7 ± 0.47	13 ± 0.82	15.7 ± 1.2	39.3 ± 2.1
V. cholerae	21.3 ± 1.2	14.3 ± 1.2	21.3 ± 0.94	25.7 ± 1.2	14.7 ± 1.2	17.3 ± 2.1	37.7 ± 2.9

Table 3: The brine shrimp lethality bioassay for petroleum ether (Pt), chloroform (Ch) and methanolic (Mt) extract of *A. cucullata* and standard vincristine sulfate.

Plant parts	Solvent system	LC_{50} value (µg/ml)	Regression equation	R^2
Bark	Pt	10	y = 12.34 + 37.51x	0.962
	Ch	2.301	y = 39.98 + 27.65x	0.834
	Mt	7.28	y = 18.92 + 36.05x	0.930
Leaf	Pt	1.308	y = 52.59 + 22.19x	0.784
	Ch	1.98	y = 41.98 + 26.94x	0.807
	Mt	2.14	y = 40.98 + 27.30x	0.824
Vincristine sulfate	----------	0.128	y = 64.64 + 16.39x	0.821

rate observed in leaf part of *A. cucullata*. Mortality rate was considered outstanding as obtained LC_{50} values were less than 250 µg/ml which indicates presence of bioactive compounds in the extracts.[8]

DISCUSSION

In determining free radical scavenging activity both bark and leaf extracts of petroleum ether and chloroformic solvent system exhibited very poor scavenging activity if compared to standard quercetin (IC_{50} value at 6.64 µg/mL); that could be attributed due to absence of active antioxidant compounds[17] and influence of extracting solvent system.[18] While presence of polyphenols and tannins on leaves[11] could be responsible for moderate scavenging activity[19] in methanolic leaf extracts.

Polyphenols are the most active antioxidant derivatives in plants.[20] Total phenolics and flavonoids content results obtained in the present study revealed that the level of these compounds in the extracts were considerable. Therefore extracts with good amount of phenolic compounds but weaker scavenging activity could be due to the fact that the free radical scavenging assay was a combined activity of a wide range of compounds,[21] absence of only flavonoids with particular hydroxyl position in the molecule[19] and finally radical scavenging activity is not only specific to presence of polyphenol groups or flavonoids.[22,23]

Reducing capacity is associated with antioxidant activity.[24] Presence of active reductants in methanolic extracts may be responsible for its good reducing ability[25] as well as moderate antioxidant activity. While influence of solvent systems may attribute towards weak reducing ability[18] as well as poor scavenging activity.

The findings of antibacterial study in the present investigation were in good accordance with this study.

The cytotoxic activity exhibited by the solvent fractions was promising and clearly indicates presence of potent bioactive compounds. Preliminary cytotoxicity of ethanolic stem and leaf extracts of *A. cucullata* was screened previously and potential brine shrimp lethality was reported (LC_{50} value around 10 µg/mL) which also suggests its possible antitumor, antibacterial or pesticidal activities.[26,27]

CONCLUSION

In conclusion, the results of antioxidant, antibacterial and brine shrimp lethality bioassay screening exhibited by the extractives of *A. cucullata* indicate the presence of bioactive principles in the extractives, especially consistent with its traditional uses in treating diseases like diarrhea and inflammation as well as showing CNS depressant activity. Four complementary antioxidant screening methods suggested moderate antioxidant activity in methanolic extracts of *A. cucullata* and further studies are required to isolate and characterize active principles with potential antioxidant properties.

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CONFLICT OF INTEREST

The authors declare no conflict of interests.

SUMMARY

- Methanolic extract of *A. cucullata* leaf showed moderate antioxidant activity.
- Both bark and leaf extractives produced maximum zone of inhibition against stomach disorder and skin disease causing bacteria.
- Both bark and leaf extracts exhibited strong cytotoxic activity.

ABBREVIATIONS USED

DPPH: 2,2-diphenylpicrylhydrazyl; IC_{50}: The half maximal inhibitory concentration; LC_{50}: The half maximal lethal concentration; FC: Folin–Ciocalteu; DMSO: Dimethyl sulfoxide, *S. dysentry : Shigella dysenteriae; S. flexneri : Shigella flexneri; S. typhi : Salmonella typhi; E. coli : Escherichia coli; P. aeruginosa : Pseudomonas aeruginosa; V. cholerae : Vibrio cholerae*.

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