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Evaluation of antimicrobial and cytotoxic potentials of *Syzygium samarangense* (Blume) merr. & l. m. perry stem bark extract

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Abstract
Objective: Now a days, one of the major public health concerns is the development of antimicrobial resistance to synthetic drugs. Medicinal plants, for their various pharmacological properties considered as important source for drug discovery. *Syzygium samarangense* (Blume) Merr. & L. M. Perry, an ethnomedicinal plant is used traditionally in bronchitis, asthma, diabetes mellitus, inflammatory disorder etc. Very few works have been done on the stem bark of this plant for its biological activities so far. This study investigates the antimicrobial and cytotoxic activities of three different solvent extracts of *S. samarangense* stem bark.
Methods: Three fractionates of *S. samarangense* stem bark extract were prepared by cold maceration and sequential solvent extraction method using n-hexane, dichloro-methane (DCM) and methanol (MeOH) as solvent. *In vitro* antimicrobial activity, minimum inhibitory concentration (MIC) and cytotoxic activity were determined by using agar diffusion assay, broth dilution method and brine shrimp lethality test respectively.
Results: The diameter of inhibition zone ranged from 8.37±0.01 to 19.53±0.02 mm for bacteria and 7.71±0.01 to 13.40±0.01mm for fungi. Among three solvents, the MeOH soluble fractionate of *S. samarangense* stem bark exhibited largest zone of inhibition to *Staphylococcus aureus*, *Escherichia coli* and *Salmonella typhi*. The MIC values of the plant extracts ranged from 64 to 512 µg / ml. *In vitro* cytotoxic studies revealed significant lethality of the MeOH extract of the plant to brine shrimps with LC_{50} 30.031 µg / ml compared to the standard.
Conclusion: The stem bark extract of *S. samarangense* have demonstrated remarkable antimicrobial and good cytotoxic activities. Thus, it may possibly be a candidate of antibiotic agent in combating infectious diseases. Further study is needed to identify the bioactive phytomolecules for the development of new, and safe drugs against resistant microbes.

Keywords: *Syzygium samarangense*, antimicrobial, disc diffusion method, cytotoxicity, brine shrimp lethality

1. Introduction
In last few decades, bacteria, fungi, parasites, and viruses are the most common causes of infectious diseases. Prevention and treatment of this infectious diseases have led to great attention in medicine [1]. Antibiotics are one of the most important drugs in fighting the bacterial infections in human. However, the health benefits of antibiotics are under threat due to the emergence of drug-resistant bacteria [2]. Thus, it is necessary to investigate new drugs with less resistance and less side effects that would be derived from natural sources.
Brine shrimp lethality bioassay is a simple, and reliable method for the assessment of bioactivity from natural sources [3]. *Artemia salina*, the brine shrimp is an invertebrate component of the fauna of saline aquatic and marine ecosystem [4]. In laboratory, it can be used to explore the toxicity by the determination of the median lethal concentration LC_{50} that have been reported for a series of toxins and plant extracts. A good relationship has been found with brine shrimp lethality test to detect antitumor compounds in plant extracts [5].
Plant is an important source of medicine and plays a vital role in the treatment of various ailments in human all over the world [6, 7]. Medicinal plants containing various phytomolecules, play a therapeutic role in the body system. In earlier report, the presence of alkaloids, flavonoids, glycosides, terpenoids, saponins, tannins, steroids, volatile oils etc. in medicinal plants are responsible for the proposed therapeutic effects [8].
Syzygium samarangense (Blume) Merr. & L. M. Perry, a tropical tree is native to Indonesia, Indochina, Malaysia and belonging to the family Myrtaceae. It is widely cultivated in tropical country; Bangladesh, India, Thailand, Srilanka and commonly known as Jamrul or Amruj tree

in Bangladesh [9]. Fruits commonly known as wax apple, java apple, rose apple, love apple; are available in summer. Different parts of *S. samarangense* such as leaves, roots, bark, fruit have potential medicinal values for the treatment of bronchitis, asthma, diabetes mellitus, and inflammatory syndromes [10].

In the literature search, Moneruzzaman *et al*. [11], reported that phytochemicals in the *S. samarangense* fruits showed antibiotic action against *Staphylococcus aureus*, *Candida albicans* and *Mycobacterium smegmatis*. It was also found that leaves and seeds of *S. samarangense* have extensive antimicrobial activities against some pathogenic microorganisms such as *Pseudomonas aeruginosa*, *Klebsiella pneumoniae* and *Cryptococcus neoformans* etc. [12]. This plant has valuable therapeutic potentials including anti hyperglycemic, antioxidant, anti-mutant, anticancer, antidiarrheal, and immunostimulant activities [9, 13-16]. The leaves of this plant contain a huge number of phytomolecules such as flavonones, flavonol glycosides, proanthocyanidins, anthocyanidins, ellagitannins, triterpenoids, chalcones, volatile terpenoids etc. [17]. However, research areas, especially its antimicrobial, and cytotoxic profile of the stem bark of this plant, require more in-depth studies. Therefore, the present study was designed to investigate the antimicrobial and cytotoxic properties of the crude extract of *S. samarangense* stem bark.

2. Material and Methods

2.1 Collection of plant materials

The fresh barks of *S. samarangense* was collected during the month of November 2021, from the village Pandit gram, situated near the Natore city of Bangladesh and identified by an expert taxonomist, Department of Botany, University of Rajshahi, Rajshahi-6205, Bangladesh.

2.2 Extraction of plant materials

The collected fresh barks of *S. samarangense* were first washed with water, cut into small pieces and then air dried for 12 days. The dried barks were pulverized into coarse powder by using grinding machine. The ground powder (847 gm) was kept under cold maceration and extracted sequentially with n-hexane (2.5 liters), dichloro-methane (DCM; 2 liters), methanol (MeOH; 2 liters) according to the increasing polarity index of each solvent. Maceration and extraction were carried out in an air tight clean flat-bottomed container for 12 days at room temperature (23±5) °C with occasional stirring and shaking [18]. Cotton plug and a Whatman No.1 filter paper (Whatman Ltd., England) were used to filter the extract. The filtrate was placed in a vacuum rotary evaporator at 40 - 50°C to afford a dry adhesive mass. The crude extracts so obtained (n-hexane; 35 gm, DCM; 31 gm, MeOH; 27 gm) were stored at 4 °C for analysis.

2.3 Bacterial and fungal strains used

The test bacterial strains were ten in number comprising of four gram (+ve); (*Streptococcus-β-haemolyticus*, *Staphylococcus aureus*, *Bacillus cereus*, and *Bacillus megaterium*), and six gram (-ve); (*Escherichia coli*, *Pseudomonas aeruginosa*, *Shigella dysenteriae*, *Shigella boydii*, *Salmonella typhi*, and *Klebsiella pneumoniae*). The antifungal activity was tested against four pathogenic fungi (*Aspergillus Niger*, *Candida albicans*, *Fusarium oxysporum*, and *Aspergillus flavus*). These microorganisms were collected from the Enteric Microbiology Laboratory, ICDDR, B, Dhaka, Bangladesh. From the stock, the organisms were sub cultured in sterile nutrient broth medium (Difco Laboratories, P^H 6.2) and incubated for 18-24 hrs at 37 °C before the day of experiment.

2.4 Disc diffusion assay

In vitro antibacterial and antifungal activities of each separate n-hexane, DCM, and MeOH soluble fraction of the plant stem bark extract was examined by using agar disc diffusion method [19]. The antibacterial and antifungal activities were investigated against ten pathogenic bacteria (four gram-positive and six gram-negative) and four fungi. To determine zone of inhibition, kanamycin (30 µg / disc, Hi-media, India), ampicillin (30 µg / disc, Hi-media, India) and ketoconazole (20 µg / disc, Hi-media, India), griseofulvin (20 µg / disc, Hi-media, India) were used as standard antibiotics for comparison of antibacterial and antifungal activities respectively. Nutrient agar media (Difco Laboratories, P^H 7.2), and sabouraud dextrose agar media (Bio life Vole Monza, P^H 5.6) were distributed in sterilized petridishes for pathogenic bacterial and fungal strains [20]. Filter paper discs (6 mm diameter), impregnated with 10µl of each crude extract were placed on an agar growth medium containing microorganisms. Using only the respective solvent, negative controls were prepared. These plates were kept 24 hrs at low temperature (4 °C) for maximum diffusion of the test materials and antibiotics and then incubated 24 hrs at 37 ºC for proper growth [21]. The sensitivities of the microorganisms to the plant extracts were determined by measuring the sizes of clear distinct inhibitory zones around the discs on the agar surface.

2.5 Determination of Minimum Inhibitory Concentration (MIC)

Minimum inhibitory concentration (MIC) was determined by the serial tube dilution techniques or turbidimetric techniques against all pathogenic bacteria according to Roland R (1982) [22]. Each fractionates of crude extract (n-hexane, DCM, and MeOH) was dissolved in 2 ml distilled water separately. At first, sample solution was prepared at 512µg / ml concentration in nutrient broth medium and then serially diluted to 256µg / ml, 128µg/ml, 64µg / ml, 32µg / ml, 16µg / ml, 8µg / ml, 4µg / ml, 2µg / ml, 1µg / ml in test tube consecutively. The same procedure was followed for the preparation of standard antibiotics; kanamycin and ampicillin. A tube containing only nutrient broth media served as control. All the tubes were then inoculated with a loop of microorganisms (≈ 10 µl) and incubated at 37 °C for 18 hrs. After incubation, the tubes were then examined by observing turbidity which is a sign of bacterial growth. The tube without visible growth was taken as the concentration at which multiplication of the organism was inhibited and marked as minimum inhibitory concentration (MIC). The concentrations of each crude extract in such tubes were recorded as MICs.

2.6 Brine shrimp lethality bioassay

The method of Meyer *et al*. (1982) was used to test brine shrimp toxicity screening of three different fractionates (n-hexane, DCM and MeOH) of the crude extract [23]. Brine shrimp eggs (*Artemia salina*) were collected from University of Dhaka, Bangladesh as a gift sample for the research work. Brine shrimps were hatched using brine shrimp eggs in a vessel filled with sterile artificial sea water (38 gm. of sea salts, sodium chloride in 1000 ml of distilled water, p^H 8.5 is adjusted with 1N NaOH) under a constant light source (60 W lamp), 24-28 °C temperature and aeration for 48 hrs. The

active shrimps called nauplii were collected and used for the assay. The test samples were prepared by dissolving each fractionate of extract in dimethyl sulfoxide (DMSO; not more than 50 µl in 5 ml solution) with sea water to obtain concentrations of 5, 10, 20, 40, and 80 µg / ml. A vial containing 50 µl DMSO diluted to 5 ml was considered as a negative control and standard vincristine sulphate as positive control. Using glass capillary tube 10 nauplii were added to each experimental vial. The number of dead nauplii were counted after 24 hrs with the help of magnifying glass. The percentage mortality of brine shrimp nauplii was determined from the number of dead nauplii. The resulting concentration-mortality data were transformed to Microsoft Excel statistical analysis to assess the lethality concentration, LC_{50} values of the plant extracts [24].

3. Results and Discussion

3.1 Antimicrobial activities of *S. samarangense* stem bark

Antibacterial and antifungal activities of three differential extracts (n-hexane, DCM, and MeOH) of *S. samarangense* stem bark were determined in terms of zone of inhibition of bacterial and fungal growth respectively. The results of the antibacterial and antifungal activities are showed in Tables 1 and 2. In case of antibacterial activity, the measured inhibition zone in mm ranged from 8.37±0.01 to 19.12±0.01 for gram (+ve) bacteria and 9.15±0.01 to 19.53±0.02 for gram (-ve) bacteria (Table 1). Among three solvents, the MeOH soluble fractionate of *S. samarangense* stem bark exhibited largest zone of inhibition while n-hexane and DCM fractionates showed less and moderate zones.

Table 1: Inhibition zone of antibacterial activities of three fractionates extract (n-hexane, DCM & MeOH) of *S. samarangense* stem bark (values were means of three replicates (n = 3) ± standard error)

Bacterial strains tested	Inhibition zone in diameter (mm) of three fractionates (500 µg / disc)			Ampicillin (30 µg / disc)	Kanamycin (30 µg / disc)
	n-hexane	DCM	MeOH		
Gram (+ve)					
Streptococcus β-haemolyticus	10.73±0.04	12.28±0.01	16.11±0.03	26.33±0.01	24.95±0.02
Staphylococcus aureus	13.15±0.01	15.25±0.02	19.12±0.01	25.21±0.10	26.24±0.34
Bacillus cereus	11.23±0.01	16.60±0.03	18.12 ± 0.02	23.51±0.04	23.61±0.01
Bacillus megaterium	8.37±0.01	14.25±0.06	17.08 ± 0.01	25.41±0.33	26.93±0.35
Gram (-ve)					
Escherichia coli	13.51±0.03	17.21±0.01	19.53±0.02	26.11±0.17	23.50±0.01
Pseudomonas aeruginosa	10.56±0.03	14.13±0.04	16.23 ± 0.01	21.11±0.17	25.52± 0.13
Shigella dysenteriae	9.15±0.01	11.29±0.04	15.01±0.07	25.31±0.03	24.11±0.01
Shigella boydii	10.13±0.01	16.12±0.03	14.71±0.32	22.11±0.02	26.01±0.03
Salmonella typhi	14.70±0.03	16.11±0.03	19.20±0.05	25.02±0.02	27.37±0.07
Klebsiella pneumoniae	12.23±0.04	17.13±0.05	18.41±0.07	23.13±0.21	25.23±0.01

The gram (+ve) *Staphylococcus aureus* (19.12±0.01mm), gram (-ve) *Escherichia coli* (19.53±0.02mm) and *Salmonella typhi* (19.20±.05mm) were more sensitive than gram (+ve) *Bacillus cereus* (18.12±0.02mm) and gram (-ve) *Klebsiella pneumoniae* (18.41±0.07mm). N-hexane fractionate of the extract was less active against the gram (+ve) *Bacillus megaterium* with the inhibition zone of 8.37±0.01mm.

The data presented in Table 2 for antifungal activities revealed that three solvent extracts (n-hexane, DCM and MeOH) of *S. samarangense* stem bark were active against all the tested fungi. The diameter of inhibition zone ranged from 7.71±0.01 to 13.40±0.01mm for three solvent extracts as compared to the standard ketoconazole and griseofulvin. MeOH fractionate was more effective against *Candida albicans* (13.40±0.01mm) and *Aspergillus flavus* (12.80±0.02mm) than *Aspergillus Niger* (10.25±0.03mm) and *Fusarium oxysporum* (11.31±0.01mm).

Table 2: Inhibition zone of antifungal activities of three fractionates extract (n-hexane, DCM & MeOH) of *S. samarangense* stem bark (values were means of three replicates (n = 3) ± standard error)

Fungal strains tested	Inhibition zone in diameter (mm) of three fractionates (500 µg / disc)			Ketoconazole (20 µg / disc)	Griseofulvin (20 µg / disc)
	n-hexane	DCM	MeOH		
Aspergillus niger	9.10±0.02	9.65±0.03	10.25±0.03	17.10±0.01	18.25±0.04
Candida albicans	9.55±0.01	10.20±0.02	13.40±0.01	18.15±0.05	19.10±0.02
Fusarium oxysporum	8.80±0.03	10.55±0.02	11.31±0.01	20.75±0.04	20.30±0.32
Aspergillus flavus	7.71±0.01	8.10±0.03	12.80±0.02	19.21±0.02	18.0±0.03

3.2 Minimum inhibitory concentration (MIC) values of bacterial strains

The evaluation of MIC (minimum inhibitory concentration) values of the selected bacterial strains were recorded in Table 3. The MIC values of the plant extracts ranged from 64 µg / ml to 512 µg/ml. The result showed that MeOH fractionate of *S. samarangense* stem bark had lowest MIC values; 64 µg / ml to *Staphylococcus aureus*, *Bacillus cereus*, *Escherichia coli*, *Shigella dysenteriae*, and *Klebsiella pneumoniae*. On the other hand, highest MIC values; 512 µg/ml were exhibited for n-hexane fractionate to *Streptococcus β-haemolyticus*, *Bacillus cereus*, *Pseudomonas aeruginosa*, *Shigella dysenteriae*, and *Klebsiella pneumoniae* strains. In the meantime, DCM soluble fractionate of the stem bark had moderate MIC values for these bacteria. The high MIC values indicate that the plant extracts are less active on some bacteria, while the low values for other bacteria is an indication of the more efficacy of the plant extracts.

Table 3: Minimum inhibitory concentrations (MICs) of three fractionates extract (n-hexane, DCM & MeOH) of *S. samarangense* stem bark.

Bacterial strains tested	MIC values (µg / ml) of three fractionates			Ampicillin (µg / ml)	Kanamycin (µg / ml)
	n-hexane	DCM	MeOH		
Gram (+ve)					
Streptococcus β-haemolyticus	512	256	128	16	32
Staphylococcus aureus	256	256	64	32	16
Bacillus cereus	512	128	64	16	8
Bacillus megaterium	256	128	128	16	8
Gram (-ve)					
Escherichia coli	256	256	64	16	8
Pseudomonas aeruginosa	512	256	128	32	16
Shigella dysenteriae	512	128	64	16	8
Shigella boydii	256	128	128	32	16
Salmonella typhi	256	256	128	32	8
Klebsiella pneumoniae	512	128	64	16	16

Depending on the solubility or polarity, different solvents have the capacity to extract different phyto constituents from the plant. The antimicrobial properties of the medicinally active plants may be due to the presence of different bioactive compounds in the extracts, which are known to act by a different mechanism [25, 26]. Phyto constituents such as tannins, flavonoids, alkaloids and several other biomolecules act as defense mechanisms against microorganisms [27]. One previous study evaluated the antimicrobial effect of the volatile oil from the leaf of *S. samarangense* on *Escherichia coli* [28]. Another study investigated significant antibacterial activities of *S. samarangense* fruits extract against two gram-positive bacteria (*Staphylococcus aureus*, *Bacillus cereus*) and two gram-negative bacteria (*Escherichia coli*, *Salmonella enterica*) [29]. Current observation on *S. samarangense* stem bark extracts showed similar results for antibacterial activities except *Salmonella enterica*. The results of antimicrobial screening tests demonstrated that among three, the MeOH fractionate showed significant inhibitory action against bacteria and fungi followed by DCM and n-hexane extract. So far, our literature review, this is the first report on *S. samarangense* stem bark extracts for the exploration of antimicrobial activities.

3.3 Cytotoxic activities through brine shrimp lethality assay

The results of brine shrimp lethality assay are represented in Table 4 and depicted in Fig 1 (a, b, c). In the bioassay, there was a gradual increase in the number of dead nauplii with the increase in the concentration of each extract. Maximum mortalities were found at 80 µg / ml concentration

Table 4: Brine shrimp lethality bioassay data for three fractionate extract (n-hexane, DCM & MeOH) of *S. samarangense* stem bark and standard vincristine sulphate

Test samples	Conc. µg / ml	Log of conc.	No. of nauplii taken in each vial	No. of nauplii dead			Average No. of nauplii dead	Percent (%) of mortality	LC_{50} µg / ml
				V1	V2	V3			
n-hexane extract	5	0.69	10	2	2	2	2.000	20.00	39.014
	10	1.00	10	4	3	2	3.000	30.00	
	20	1.30	10	5	4	4	4.333	43.33	
	40	1.60	10	6	6	5	5.667	56.67	
	80	1.90	10	8	7	7	7.333	73.33	
DCM extract	5	0.69	10	3	2	1	2.000	20.00	33.907
	10	1.00	10	3	2	4	3.000	30.00	
	20	1.30	10	5	3	6	4.667	46.67	
	40	1.60	10	6	5	7	6.000	60.00	
	80	1.90	10	8	7	7	7.333	73.33	
MeOH extract	5	0.69	10	3	2	2	2.333	23.33	30.031
	10	1.00	10	4	3	4	3.667	36.67	
	20	1.30	10	6	4	5	5.000	50.00	
	40	1.60	10	6	5	8	6.333	63.33	
	80	1.90	10	8	7	9	8.000	80.00	
Vincristine sulphate (standard)	5	0.69	10	3	3	4	3.333	33.33	6.307
	10	1.00	10	5	4	6	5.000	50.00	
	20	1.30	10	7	5	9	7.000	70.00	
	40	1.60	10	10	10	10	10.000	100.00	
	80	1.90	10	10	10	10	10.000	100.00	
DMSO	5	0.69	10	0	0	0	0	0	-------
	10	1.00	10	0	0	0	0	0	
	20	1.30	10	0	0	0	0	0	
	40	1.60	10	0	0	0	0	0	
	80	1.90	10	0	0	0	0	0	

for each extract whether least mortalities were at 5µg/ml. The lethality concentration 50 (LC_{50}) of the standard vincristine sulphate was found to be 6.307 µg / ml. Compared to the standard, MeOH extract of *S. samarangense* stem bark showed low LC_{50} of 30.031 µ g / ml indicating good cytotoxic properties of plant extracts. On the other hand, n-hexane

extract has relatively larger LC_{50}value, 39.014 µg / ml than DCM extract with LC_{50} 33.907 µg / ml.

Brine shrimp lethality assay is a simple, and inexpensive method for evaluating bioactivity of plants extracts which correlates with cytotoxic and anti-tumor properties [30, 31]. Previous investigation revealed the identification of four cytotoxic compounds and their cytotoxic activities from the methanolic extracts of the pulp and seeds of *S. samarangense* fruits against the SW-480 human colon cancer cell line [13]. Similarly in the present study, the MeOH extract of *S. samarangense* stem bark showed potent cytotoxicity for brine shrimps. There was no previous report on anticancer activity of the stem bark part of this plant extract or its isolated compounds. Therefore, in order to discover potent anticancer agents, methanolic partition ate of crude extract of *S. samarangense* stem bark needs to be evaluated in future.

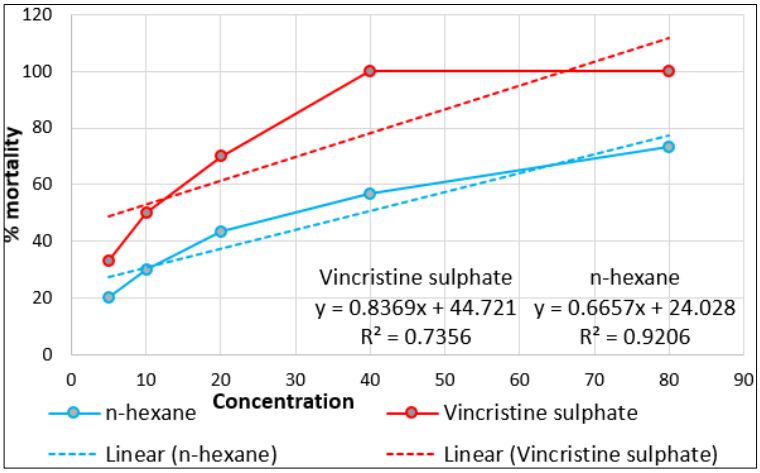

Fig 1: (a) Brine shrimp lethality bioassay of n-hexane fractionate of *S. samarangense* stem bark extract in comparison to the standard vincristine sulphate

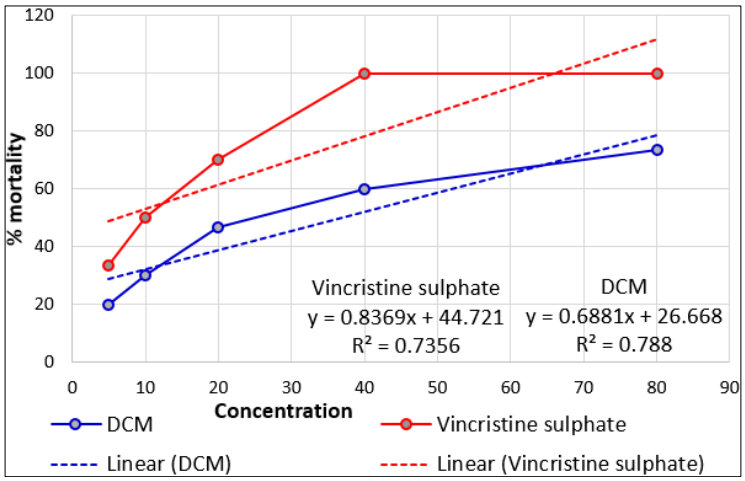

Fig 1: (b) Brine shrimp lethality bioassay of DCM fractionate of *S. samarangense* stem bark extract in comparison to the standard vincristine sulphate

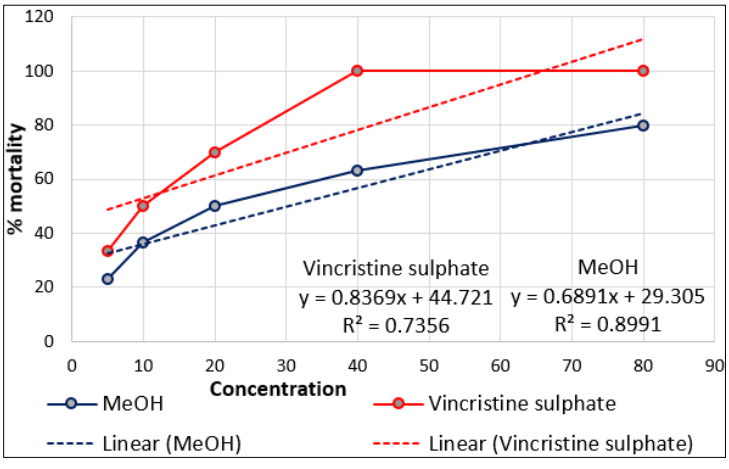

Fig 1: (c) Brine shrimp lethality bioassay of MeOH fractionate of *S. samarangense* stem bark extract in comparison to the standard vincristine sulphate

4. Conclusion

In recent years, antibiotics are used routinely as an essential part for the prevention and treatment of bacterial diseases. However, effectiveness of these synthetic drugs is gradually decreasing due to the development of bacterial resistance. This phenomenon has becoming as one of the most serious threats to human health all over the world. Thus, introducing natural components possessing antimicrobial properties may serve as an alternative for antibiotics. In this sense, the phyto constituents present in the stem bark extract of *S. samarangense* may provide a potential candidate and have become an emerging research interest in future. In addition, the significant lethality of the MeOH soluble fractionate of *S. samarangense* stem bark to brine shrimps is an indicative of the presence of potent cytotoxic compounds which needs further investigation.

5. Conflicts of interest

The authors declare no conflict of interest.

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