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Phytochemical and biological investigation of VLC fraction of *Senna alata* leaves

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Abstract
The objective of this work was phytochemical and biological investigation of VLC fraction of *Senna alata* leaves found in Bangladesh. The crude methanolic extract was fractionated by vacuum liquid chromatography by different five polar solvent. The resulted fractions were again fractionated by vacuum liquid chromatography by same five different polar solvent. We investigated the presence of polyphenols, flavonoids and other types of compounds by thin layer chromatography. *In vitro* antioxidant effects were measured by DPPH scavenging assay. *In vitro* anti- diabetic assay was carried out by glucose uptake in yeast cells. Disc diffusion assay was performed to show the antibacterial effect using gram positive, gram negative strains of bacteria and fungi. Receptor binding activities was performed by hemagglutination inhibition assay. VLC fraction of leaves contain flavonoids and other biologically active compounds. The extract showed antioxidant and anti-diabetic activity. The extract also showed antibacterial activities against several strains of bacteria. It also showed hemagglutination inhibition activities in human blood cells. Therefore, leaves of *Senna alata* may be considered as a source of various health benefits.

Keywords: *Senna alata,* DPPH scavenging assay, hemagglutinin, methanolic extract, VLC fraction.

1. Introduction
Nature has been a source of medicinal agents for thousands of years and an impressive number of modern drugs have been isolated from natural resources. Traditional medicine is an important source of potentially useful new compounds for the development of chemotherapeutic agents. The essential values and uses of some plants have been worked out and published, but many of them remain unexplored to date. Therefore, there is a necessity to explore their uses and to conduct pharmacognostic and pharmacological studies to discover their medicinal properties [1].

Medicinal plants have been used in traditional health care systems since prehistoric times and are still the most important healthcare source for the most of the world's population. The World Health Organization (WHO) has estimated that more than 75% of the world's total population depends on herbal drugs for their primary healthcare needs [2]. In the recent years, research on medicinal plants has attracted a lot of attentions globally. Large body of evidence has accumulated to demonstrate the promising potential of medicinal plants used in various traditional, complementary and alternate systems of treatment of human diseases [3].

Senna alata (Linn.) Roxb. or Chum-het-thet, former *Cassia alata* Linn., family Fabaceae (Leguminosae), is generally known as ringworm tree, Christmas candle, candlestick or candle bush [4]. It is an ornamental shrub that is widely distributed in the wild in all parts of Bayelsa state especially when dry season is approaching. This shrub is found in other southern part of the country including the south-east and south- west [5]. *Senna alata* is traditionally used as purgative, anthelmintic, antipyretic, cathartic, laxative, vermifuge, diuretic, detoxing, colon cleansing, body detoxing [6]. Phytochemical screening of the *Senna alata* leaves showed positive results for saponins (1.22%), flavonoids (1.06%), cardiac glycosides (0.20%), cardenolides and dienolides (0.18%), phenolics (0.44%) and alkaloids (0.52%) [7].

Here we examined the antioxidant, antidiabetic, antibacterial, hemagglutination inhibition activity of the VLC fraction of *Senna alata* leaves.

2. Methodology
2.1 Plant collection
The plant sample was collected from Jhalokathi District, under Barisal division on 9th October, 2014 and identified by the taxonomist of the Bangladesh National Herbarium,

Mirpur, Dhaka as *Senna alata*. A voucher specimen of the plant has been deposited (Accession No.: 41563) in the herbarium for further reference.

2.2 Extraction of the plant material

Sun-dried and powdered plant material (1690 g) was extracted with methanol. The extracts were concentrated with a rotary evaporator (IKA, Germany) at low temperature (40-50 °C) and reduced pressure. A layer of oil was found in the methanolic extract, which was collected. The extracts were stored at 4 °C until used. Methanolic extrct was subjected to Vacuum Liquid Chromatographic procedure using DCM (100%), n-hexane (100%), ethyl acetate (100%) and methanol (100%) solvent systems. Then four fractions were obtained which were concentrated to dryness. Then the resulted fractions were again subjected to Vacuum Liquid Chromatographic procedure using DCM (100%), n-hexane (100%), ethyl acetate (100%) and methanol (100%) solvent systems.

2.3 TLC Analysis

The VLC fractions were analyzed by performing TLC to determine the composition of extract. TLC was done using three solvent systems. After development of TLC plates, they were exposed to UV light. For charring the plates were sprayed with 10% sulphuric acid solution, dried and then heated to 80-90 °C. This allowed the spots to be visible. For detection of flavonoids the plates were dipped into 0.04% DPPH solution and dried while keeping in a dark place. For detection of polyphenols the plates were washed with Folin-ciocalteu reagent and dried.

2.4 DPPH Free Radical Scavenging Assay

The DPPH (1, 1- diphenyl-2-picryl hydrazyl) radical scavenging method was used for the determination of the antioxidant capacity of the sample. Different concentrations of the methanol extract of *Senna alata* leaves (500, 250, 125, 62.5, 31.25 µg/ml, in methanol) were prepared and 100 µl of DPPH solution was added. Different concentrations of L-Ascorbic acid (31.25-500 µg/ml) were used as the standard antioxidant. After 30 min at room temperature, the absorbance values were measured at 517 nm on a spectrophotometer and expressed into percentage of antioxidant activity using the following equation: DPPH antiradical scavenging capacity (%) = (Absorbance of blank – Absorbance of sample) × 100/Absorbance of blank. DPPH solution plus methanol was used as a control [8]. The results were expressed as mean ± standard deviations.

2.5 Glucose uptake in Yeast cells

Yeast cells were prepared according to the method of Yeast cells briefly, commercial baker's yeast was washed by repeated centrifugation (3,000 rpm; 5 min) in distilled water until the supernatant fluids were clear and a 10% (v/v) suspension was prepared in distilled water. Various concentrations of extracts (500,250, 125, 62.5, 31.25 µg/ml) were added to 1mL of glucose solution and incubated together for 10 min at 37 °C. Reaction was started by adding 100 µl of yeast suspension, vortex and further incubated at 37 °C for 60 min. After 60 min, the tubes were centrifuged (2,500 rpm, 5 min) and glucose was estimated in the supernatant. Metformin was taken as standard drug [9]. The percentage increase in glucose uptake by yeast cells was calculated using the following formula: Increase in glucose uptake (%) = (Abs sample – Abs control) X 100/ Abs sample. Where, Abs control is the absorbance of the control reaction (containing all reagents except the test sample) and Abs sample is the absorbance of the test sample. All the experiments were carried out in triplicates [10].

2.6 Antimicrobial Screening of VLC fraction

The antibacterial activity was carried out by the disc diffusion method [11] using 100µL of suspension containing ~10^3 CFU/mL of microorganism spread on nutrient agar medium (Himedia, India). Four different bacterial strains of gram positive, seven different strains of gram negative bacteria and two strains of fungi were used to carry out this assay. Dried and sterilized filter paper discs (6 mm diameter), VLC fraction of *Senna alata* leaves, a stock solution of 3 mg/ml was prepared and discs was soaked with solutions of 10µl of test samples and dried placed [12]. Standard disc of ciprofloxacin (30 µg/disc) was used as positive control. After incubation at 37 ^{0}C for 24 hours, the antimicrobial activity of the test agents was determined by measuring the diameter of zone of inhibition expressed in mm. The results were expressed as mean ± standard deviations.

2.7 Minimum Inhibitory concentration of VLC fraction of *Senna alata* leaves

The minimal inhibitory concentration (MIC) values, which represent the lowest extract concentration that completely inhibits the growth of microorganisms. The sample in question is first prepared by producing a standard stock solution then subsequently diluting it to obtain different concentration. Minimum Inhibitory concentration is carried out by using 100 µL of suspension containing ~10^3 CFU/mL of microorganism spread on nutrient agar medium (Himedia, India). S. aureus and S. cerevisiae were used to carry this assay. Dried and sterilized filter paper discs (6 mm diameter), methanol extract of egg shell, a stock solution of 3 mg/ml was prepared. The plant samples were two fold serially diluted in eppendorf tubes to get different concentrations were prepared and discs were soaked with each solutions of 10 µl of test samples were placed gently on the previously marked zones in the agar plates. After incubation at 37 ^{0}C for 24 hours, the antimicrobial activity of the test agents was determined by measuring the diameter of zone of inhibition expressed in mm. The results were expressed as mean ± standard deviations.

2.8 Hemagglutination Inhibition Assay

Hemagglutination activity of VLC fractions were tested against human erythrocyte blood group B+ (positive) with some modifications [13]. Stock solution of the test samples was prepared at concentration of 5 mg/ml and each solution was serially diluted. Fresh blood was collected from healthy persons, centrifuged and the erythrocytes were separated. 2% erythrocyte suspension was prepared in phosphate buffer (pH 7.4). 50 µl of sample was placed in the first well and then this was subsequently diluted two fold up to the 11 th well. 50 µl of the RBC suspension was added to all the wells and was incubated for one hour at 4 °C. After incubation, the results

were noted. Smooth button formation in bottom indicated negative activity, while a rough granular deposition at bottom showed positive activity. The intensity of hemagglutination was determined from the extent of deposition.

3. Results

3.1 TLC Analysis

TLC analysis was done as described in materials and methods. The plates were observed under UV light. After charring of the TLC plate with sulfuric acid spots were visualized. After being soaked into DPPH and FC solution, plate D and E showed moderate yellow color (Figure: 1) which indicated the presence of flavonoids and polyphenol in the separated compound of the VLC fractions.

3.2 DPPH Free Radical Scavenging Assay

The *in vitro* antioxidant assay performed on this plant reveals significant antioxidant potential. methanol→DCM, ethyl acetate→DCM and oil fraction of *Senna alata* leaves showed DPPH free radical scavenging activity 65.03%, 62.33%, 64.10% respectively at the highest concentration (500 µg/ml)(Table:1). This indicates that there are many compounds present in them that have antioxidant potential.

3.3 Glucose uptake in Yeast cells

DCM→DCM, oil fraction of *Senna alata* leaves increased the glucose uptake in yeast cell 71.72%, 75.04%, respectively at the highest concentration (500 µg/ml) whereas the standard (metformin) increased the glucose uptake in yeast cell 32.22% at the highest concentration (500 µg/ml)(Table:2). This result indicated that VLC fraction of *Senna alata* leaves had greater efficiency in increasing the glucose uptake by yeast cells as compared to standard drug metformin.

3.4 Antimicrobial Screening of methanol Extract

The antimicrobial activity of the VLC fraction of *Senna alata* leaves were studied against three Gram-positive (*Bacillus subtilis, Bacillus cereus, Staphylococcus aureus*) and seven Gram-negative (*Shigella dysenteriae, Shigella boydii, Vibrio parahaemolyticus, Vibrio mimicus, Salmonella paratyphi, Salmonella typhi, Pseudomonas aureus*), and one fungal strains (*Aspergillus niger*). Antibacterial and antifungal potential of extracts were assessed in terms of zone of inhibition of bacterial growth. The results of the antibacterial and antifungal activities are presented in Table 3. From the result it is observed the *Senna alata leaves* possess antibacterial and antifungal activities.

3.5 Minimum Inhibitory Concentration of VLC fraction of *Senna alata* leaves

Different concentration of VLC fraction of *Senna alata* leaves was tested upon the Gram positive (*B. subtilis*), Gram negative stains (*S. paratyphi*, V. parahaemolyticus, S. boydii) and fungi (*A. niger*) the zones of inhibition were recorded. The antibacterial activities of the extracts increased linearly with increase in concentration of extracts. The results of this experiment are presented in Table 4.

3.6 Hemagglutination Inhibition Assay

Almost all VLC fractions of *Senna alata* leaves exhibited hemagglutination inhibition activity potentially from highest concentration 250 µg/ml to 1.95 µg/ml i.e. it has potential binding capacity with human erythrocytes.

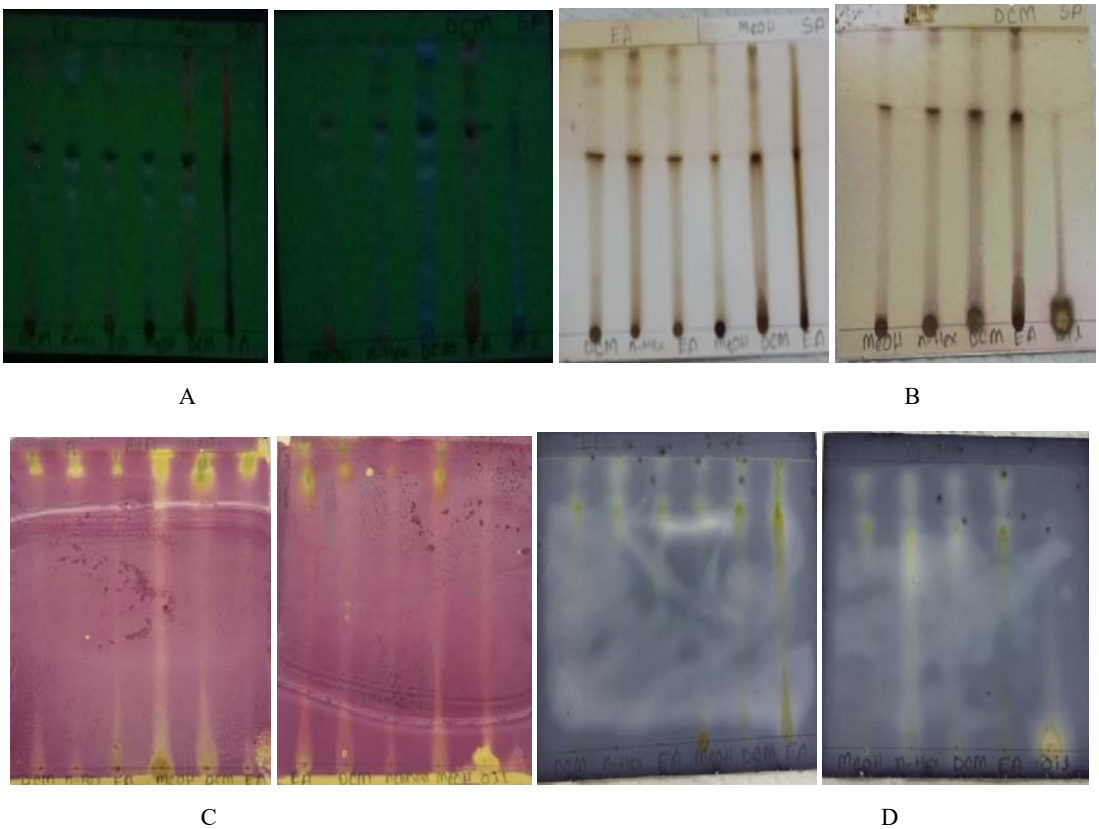
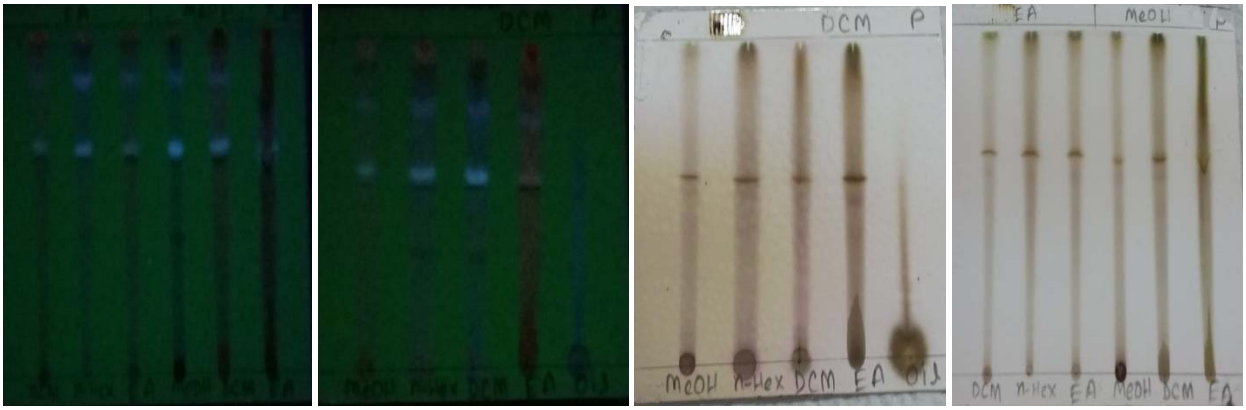
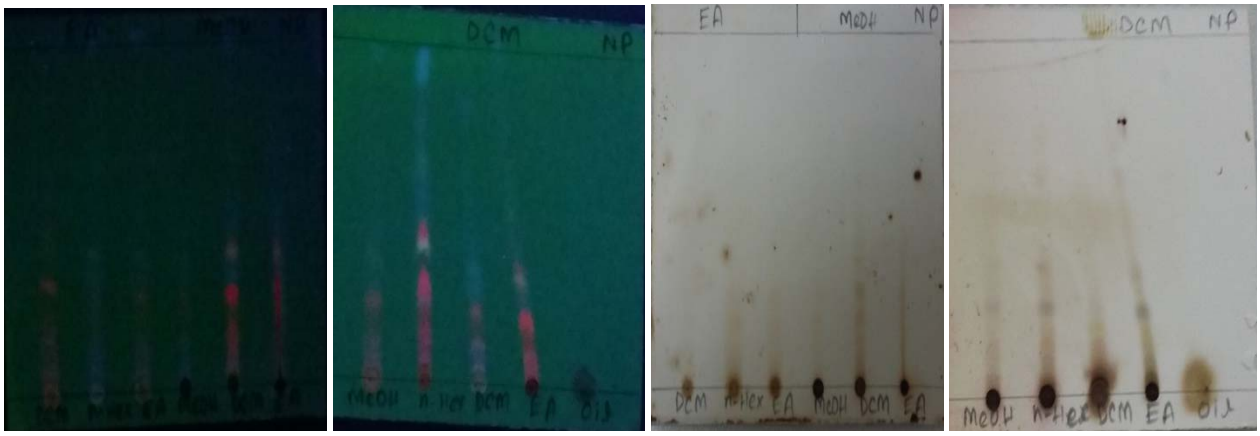


Fig 1: Separation of VLC fraction of *Senna alata* leaves using chloroform, ethyl acetate, and formic acid (5:4:1) solvent system. Key: A = Ultra-Violet view; B = Charring view; C = DPPH staining view; D =FC reagent staining view.



A B

Fig 2: Separation of VLC fraction of *Senna alata* leaves using ethyl acetate, ethanol, and water (8:1.2:0.8) solvent system.
Key: A= Ultra-Violet view; B= Charring view.



A B

Fig 3: Separation of VLC fraction of *Senna alata* leaves using benzene, ethanol, and ammonium hydroxide (9:1:0.1) solvent system.
Key: A= Ultra-Violet view; B= Charring view.

Table 1. DPPH free radical scavenging activity of VLC fraction of *Senna alata* leaves.

Concentration (µg/ml)	Me → Me	Me →DCM	Me → EA	DCM → n-hex	EA → DCM	Oil	Standard (Ascorbic Acid)
500	61.23 ± 0.27	65.03 ± 0.13	50.14 ± 0.67	61.86 ± 0.25	62.33 ± 0.45	64.10 ± 1.18	74.68 ± 0.01
250	56.17 ± 0.00	62.66 ± 0.01	46.35 ± 0.80	60.75 ± 0.51	34.65 ± 0.12	59.51 ± 0.79	67.41 ± 0.62
125	53.16 ± 0.33	46.47 ± 2.81	42.23 ± 0.95	59.17 ±0.57	13.73 ± 1.99	58.40 ± 0.52	61.70 ± 0.48
62.5	50.93 ± 0.86	36.98 ± 2.70	38.43 ± 1.09	42.08 ±1.73	----	55.06 ± 0.27	56.48 ± 0.44
31.25	47.93 ± 0.75	28.76 ± 2.10	31.96 ± 0.20	28.46 ± 1.22	----	52.20 ± 0.81	53.15 ± 0.78

Values were expressed as mean ± SD
Key: Me=Methanol, DCM=Dichloromethane, EA=Ethyl Acetate, n-hex=n-hexane

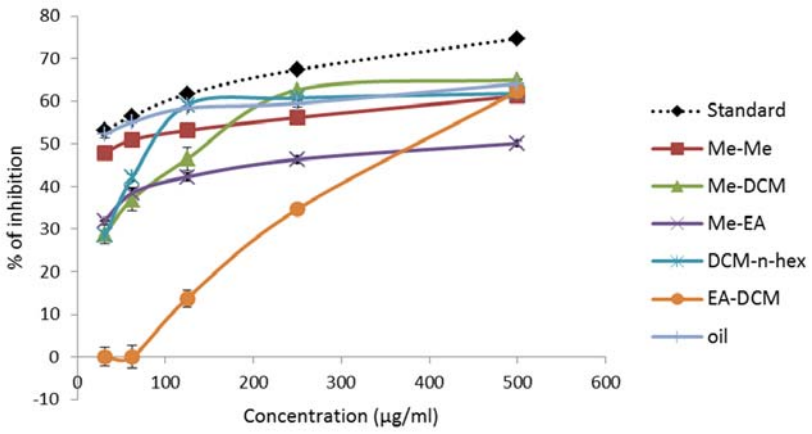


Fig 4: DPPH free radical scavenging activity of VLC fraction of *Senna alata* leaves.

Table 2: Glucose uptake in yeast cells by VLC fraction of *Senna alata* leaves.

Concentration (µg/ml)	Me → DCM	Me → EA	DCM → DCM	Oil	Standard (Metformin)
500	59.70 ± 1.33	64.11 ± 1.14	71.72 ± 0.17	85.04 ± 0.38	32.22 ± 1.07
250	42.53 ± 0.34	51.61 ± 0.83	50.21 ± 1.80	83.78 ± 0.44	26.93 ± 0.87
125	31.42 ± 0.30	35.23 ± 1.60	28.43 ± 0.54	80.24 ± 0.28	22.50 ± 2.17
62.5	21.80 ± 2.32	22.29 ± 2.74	23.67 ± 1.61	75.73 ± 1.52	17.43 ± 1.27
31.25	10.81 ± 1.80	13.92 ± 2.26	20.88 ± 1.90	61.08 ± 0.39	12.26 ± 0.78

Values were expressed as mean ± SD

Key: Me=Methanol, DCM=Dichloromethane, EA=Ethyl Acetate, n-hex=n-hexane

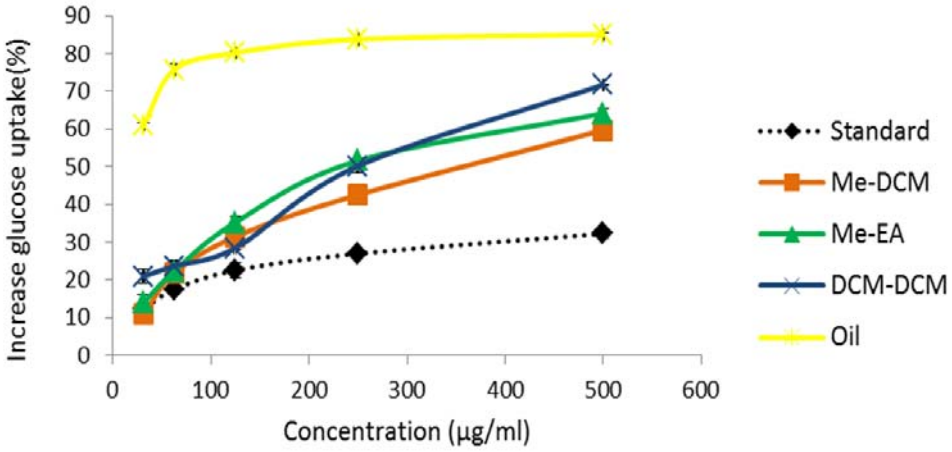


Fig 5: Glucose uptake in yeast cells by VLC fraction of *Senna alata* leaves.

Table 3: Antibacterial Activity of VLC fraction of *Senna alata* leaves on the microorganisms test.

Bacteria name	Zone of Inhibition (mm)											
	Me→ Me	Me→DC M	Me→E A	DCM→ Me	DCM → n-hex	DCM→DC M	DCM→E A	EA→DC M	EA→E A	EA→ n-hex	Oil	Contr ol
B. cereus	8 ± 0.07	12± 0.14	12 ± 0.07	9 ± 0.21	12 ± .21	11 ± 0.28	10 ± 0.71	10 ± 0.35	8 ± 0.21	9 ± 0.14	17 ± 0.28	34 ± 1.41
B. subtilis	11 ± 0.70	12 ± 0.70	12 ± 0.14	7 ± 0.07	9 ± 0.07	8 ± 0.07	9 ± 0.71	8 ± 0.14	9 ± 0.14	12 ± 0.71	21 ± 0.71	34 ± 1.13
S. paratyphi	8 ± 0.07	12 ± 0.21	12 ± 0.28	7 ± 0.14	12 ± 0.14	9 ± 0.07	12 ± 0.14	9 ± 0.21	10 ± 0.71	10 ± 0.71	17 ± 0.71	33 ± 0.71
S. typhi	6 ± 0.14	9 ± 0.14	11 ± 0.71	7 ± 0.14	10 ± 0.07	9 ± 0.07	9 ± 0.71	7 ± 0.14	9 ± 0.21	9 ± 0.71	16 ± 0.71	32 ± 0.99
V. parahaemolyticus	10 ± 0.07	10 ± 0.71	13 ± 0.21	9 ± 0.14	12± 0.07	11 ± 0.14	12 ± 0.71	11 ± 0.21	11± 0.71	11 ± 0.21	21 ± 0.35	34± 0.35
V. mimicus	8.95 ± 0.07	12.1 ± 0.14	13.85 ± 0.07	9.05 ± 0.07	11.5 ± 0.07	11.5 ± 0.07	13.5 ± 0.71	10.85 ± 0.21	11.1 ± 0.14	12.05 ± 0.71	19.5 ± 0.71	35.5 ± 2.12
E. coli	8 ± 0.14	10 ± 0.71	10± 0.07	8 ± 0.07	10 ± 0.35	7 ± 0.14	1 ± 0.21	8± 0.21	11 ± 0.35	11 ± 0.21	10 ± 0.21	34 ± 0.71
S. dysenteriae	8.05 ± 0.07	13.05 ± 0.35	13.05 ± 0.07	10.85 ± 0.21	12.15 ± 0.21	11.2 ± 0.28	12.8 ± 0.28	6.25 ± 0.21	11.75 ± 0.35	12.6 ± 0.57	13.9 ± 0.14	34.25 ± 1.06
P. aureaus	7.9 ± 0.14	10.9 ± 0.14	11.05 ± 0.07	7.15 ± 0.21	7.1 ± 0.14	6.95 ± 0.07	8.85 ± 0.21	7.35 ± 0.92	6.9 ± 0.14	6.9 ± 0.14	12.75 ± 0.35	35.5 ± 0.71
S. boydii	7.15 ± 0.21	10 ± 0.14	12.9 ± 0.14	6.95 ± 0.07	13.9 ± 0.14	13.85 ± 0.21	13.6 ± 0.57	9.9 ± 0.14	11.35 ± 0.92	11.65 ± 0.49	17.5 ± 0.71	34.25 ± 0.35
A.niger	7 ± 0.28	9.9 ± 0.14	10.9 ± 0.14	6.9 ±	10.95 ± 0.21	7.4 ± 0.85	10.85 ± 0.21	10.75 ± 0.35	10.7 ± 0.42	13.55 ± 0.64	13.8 ± 0.28	37 ± 1.41

Values were expressed as mean ± SD

Key: Me=Methanol, DCM=Dichloromethane, EA=Ethyl Acetate, n-hex=n-hexane

Table 4: MIC of the oil fraction of *Senna alata* leaves when tested with *B. subtilis, S. paratyphi* and *V. parahaemolyticus*, *S. boydii*, *A. niger*.

Concentration (µg)	100	50	25	12.5	6.25	3.12
Bacteria	**Zone of inhibition (mm)**					
Bacillus subtilis	21.5	13	11	10	6	0
Salmonella paratyphi	16.5	11	8	7	0	0
Vibrio parahaemolyticus	21.75	10	9	9	0	0
Shigella boydii	21.75	10	9	9	0	0
Aspergillus niger	13.8	8	6	6	0	0

Table 5: Hemagglutination Inhibition, Test for the VLC fraction of *Senna alata* leaves.

Sample name	Concentration (µg/ml)									
	250	**125**	**62.5**	**31.25**	**15.62**	**7.81**	**3.90**	**1.95**	**0.97**	**0.48**
Me→Me	+	+	+	+	+	+	+	+	+	-
Me→EA	+	+	+	+	+	+	+	+	-	-
Me→DCM	+	+	+	+	+	+	+	+	-	-
DCM→n-hex	+	+	+	+	+	+	+	+	+	-
DCM→Me	+	+	+	+	+	+	+	-	-	-
DCM→DCM	+	+	+	+	+	+	+	+	-	-
DCM→EA	+	+	+	+	+	+	+	+	-	-
EA→DCM	+	+	+	+	+	-	-	-	-	-
EA→EA	+	+	+	+	+	-	-	-	-	-
EA→n-hex	+	+	+	+	+	+	+	-	-	-
Oil	+	+	-	-	-	-	-	-	-	-

4. Discussions

TLC plates were seen under UV light and found different compound were separated at the the plates. Charring with H_2SO_4 in high temperature the separated compound transformed into black color. Staining the plate with DPPH solution the color of the separated compounds changed into yellow color and after staining the plate with FC-reagent the color of the separated compounds changed into bluish Such a result indicated the presence of flavonoids in the separated fractions of the extract in the semi polar mobile phase.

To evaluate the antioxidant activities of VLC fraction of *Senna alata* leaves DPPH Free Radical Scavenging Assay was used. The principle of DPPH method is based on the reduction of DPPH in the presence of a hydrogen donating antioxidant. Extracts reduce the color of DPPH due to the power of hydrogen donating ability. DPPH is one of the compounds that possess a proton free radical with a characteristic absorption, which decreases significantly on exposure to proton radical scavengers. Antioxidants may guard against reactive oxygen species (ROS) toxicities by scavenging reactive metabolites and converting them to less reactive molecules [14]. Previous study revealed that compound isolated from ethyl acetate fraction of *Senna alata* leaves possess potent antioxidant activity [3]. In our study we also found that VLC fraction of ethyl acetate showed highest DPPH anion scavenging activity compared to other fraction which was closed to standard (ascorbic acid) (table: 1).

Regulation of glucose level in the blood of the diabetic patient can prevent the various complications associated with the disease. The maintenance of plasma glucose concentration for a long term under a variety of dietary conditions is one of the most important and closely regulated processes observed in the mammalian species [15]. From the previous study, it was found that the methanol extract of leaves of *C. alata* showed potent α-glucosidase inhibitory activity. It also found that the ethyl acetate and *n*-butanol fractions displayed the highest carbohydrate enzyme inhibitory effect. [16]. the *in vitro* assays of the present study indicated that oil fraction of *Senna alata* leaves possess good anti-diabetic activity. The rate of glucose transport across cell membrane in yeast cells system is presented in Table 2. In Yeast (*Saccharomyces cerevisiae*) glucose transport takes place through facilitated diffusion. Type 2 Diabetes is characterized by the deficiency of insulin causing increased amount of glucose in blood. After the treatment of the yeast cells with these extracts, the glucose uptake was found to increase in a dose dependent manner. Results also indicated that *Senna alata* had greater efficiency in increasing the glucose uptake by yeast cells as compared to standard drug metformin.

Antimicrobial screening, oil fraction of *Senna alata* leaves showed highest activity against bacteria and fungi. The positive control was used Ciprofloxacin. A previous study revealed that different fraction of *Senna alata* leaves were showed broad spectrum of antibacterial activity [17]. The result found in our study might have correlation with this. Oil fraction was showed zone of inhibition up to 21 mm antibacterial activity at the concentrations used against *B. subtilis, V. parahaemolyticus* and other strain.

Due to the good result minimum inhibitory concentration test was carried out with oil fraction of *Senna alata* against the Gram positive (*B. subtilis*), Gram negative stains (*S. paratyphi*, *V. parahaemolyticus, S. boydii*) and fungi (*A. niger*). From the results we see that the MIC of the oil fraction of *Senna alata for S. paratyphi*, *V. parahaemolyticus, S. boydii* and *A. niger* is 12.5 µg/disk and for *V. parahaemolyticus* was 6.25 µg/disk (table: 4). Therefore, *Senna alata* may be considered as a useful source for discovering a safe and novel antimicrobial compound.

Hemagglutination inhibition assay was performed to investigate the receptor binding affinity of the compounds

present in the VLC fraction of *Senna alata* on human erythrocytes. It was observed that all the VLC fraction of *Senna alata* has potent binding affinity to the different receptors of erythrocytes and prevent agglutination. Hence the results showed possible benefits of VLC fraction of *Senna alata* as an antiviral therapeutics.

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