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In vitro Investigation of Antimicrobial, Antitumor and DPPH Reduction Capacity of the Methanolic Extract of *Scoparia dulcis*

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Authors' contributions

This work was carried out in collaboration between all authors. Authors PA and AKMRUI designed the current project, performed the experiments and wrote the manuscript. Author AYM carried out the experimental process. Author PA was also responsible for data interpretation and statistical analysis. Author SMNH helped in experiments and preparing the manuscript. Authors MNU, MS and NS participated in experiments and data collection. Author PA also edited the manuscript. All authors read and approved the final version of the manuscript. All authors read and approved the final manuscript.

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ABSTRACT

Scoparia dulcis belongs to the family Plantaginaceae. It is a flowering plant and locally common in the treatment of gastrointestinal upset and malaria. The aim of this study was to investigate the antimicrobial, antitumor and antioxidant activity of methanolic leaf extract of *S. dulcis*. The DPPH free radical scavenging assay was used to evaluate antioxidant property. The disk diffusion method

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and brine shrimp lethality bioassay were used to determine antibacterial and cytotoxic activity, respectively. In the evaluation of antioxidant property IC_{50} was found to be 196.66 µg/ml, in cytotoxicity testing, it was found that the plant extract has 29.868 µg/ml of LC_{50}. The methanolic extract of *S. dulcis* leaves also have efficiency in bacterial growth inhibition; this extract is effective against both gram negative and positive bacteria. The zones of inhibition at 500 µg/ml dose in *E. coli* and *S. aureus* culture were 20 mm and 22 mm, respectively. In thin layer chromatography analysis, presence of non-polar and polar compounds, three non-chromatophoric compounds were found. We conclude that the *S. dulcis* is a potent antibacterial, antioxidant and cytotoxic medicinal plant.

Keywords: Antimicrobial; antioxidant; antitumor; In vitro; S. dulcis.

1. INTRODUCTION

From the ancient era, it is human's nature to find cure in the herb source. This practice is still popular among people on all continents, and most of them have their own enriched prehistory. There is evidence that plants are still widely used in ethnomedicine around the world. There exist around 250,000 to 500,000 species of plants on Earth [1]. Only a small fraction of them most likely 1-10% of them are used as food by both humans and other animals. So, there is huge possibility to use plants in medical practice and remedy purposes [2].

The term antibiotic covers broad ranges of agents, which either kills or suppress microbial growth, like antimicrobials, antifungal and other compounds [3]. Waksmanin first used the term antibiotic in 1942 to describe any substance that intersects the replication of or kills microorganisms [4]. Most of today's antibiotics are either structural modification or isomerism of the 1st generation antibiotics that used to be natural compounds, for example, Penicillin, Cephalosporin, Sulfonamide, Quinolone, and so forth [5]. Plant chemicals that are usually supposed to be responsible for antibacterial effects used to have phenolic ring, alkaloid, tannins, most likely. For example, common herbs like thyme and tarragon which possess effective antibacterial, antifungal, and antiviral activities, contain caffeic acid in phytochemical list [6-9]. The mechanisms are yet not clear but are thought to be responsible for phenolic toxicity to microorganisms through the inhibition of enzymes by the oxidation, possibly through reaction with sulfhydryl groups or through other nonspecific interaction with the microbial proteins [10].

The liver is a highly sensitive organ, which plays a major role in maintenance and performance of the homeostasis in our body. It is the major organ where processes like metabolism and detoxification take place. Thus chronic exposure to drugs, environmental toxicants and other xenobiotics can lead to injury [11]. Liver disorders are one of the serious health issue, at present time in the world. Ethanol is a lipid-soluble non-electrolyte and is readily absorbed from the skin and gastrointestinal tract. It quickly diffuses to the circulatory system, and is dispersed evenly all through the body [12]. Ethanol is metabolized in the liver and persons who consume regularly and get addicted to alcohol (drinks 4 to 5 liters per day) are at risk of chronic liver diseases [13]. Moreover, both acute and chronic intake of ethanol produces cytokines in large amounts, particularly TNF-α by hepatic κ-cells, which play a major role in causing liver injury [14-16]. These results in the accumulation of hepatic lipids and lipid peroxides, and leads to auto-oxidation of hepatic cells either by acting as a pro-oxidant or by decreasing the antioxidant levels, thereby resulting in a remarkable hepatotoxicity. Lipid peroxidation by ethanol induces hepatic oxidative stress, which has been identified to be involved in the pathomechanism of Alcoholic Liver Disease (ALD) [17].

There is evidence that of the total oxygen consumed, almost 5% is converted into oxygen derived free radicles [18,19]. These free radicals are known as reactive oxygen species or ROS (e.g., O_2^-, H_2O_2, OH^-), that are formed in body as by-products of different metabolism processes and from exogenous sources. ROS molecules produce a stressed condition in the human body that causes each cell to face about 10000 hits per second [20]. If the generation of ROS exceeds the antioxidative defense of body cells, the macromolecules (like lipids, proteins, carbohydrates) of the human body are then attacked by free radicals and different disease condition appear [21-23]. Free radicals are responsible for pathogenic conditions of degenerative diseases like Alzheimer's, and they are also involved in the consequence of diabetes, cardiovascular disease, nephrotoxicity,

neurotoxicity and so on [24]. Many plants contain molecules like vitamin C and E, flavonoids, carotenoids, phenolic compounds etc. that have the ability to prevent oxidation and remove excess free radicals from the body [25].

Scoparia dulcis is a member of the Plantaginaceae family. It is an Ethnobotanically very important and widely used plant. In some parts of Bangladesh, this flowering plant is known as weed. *S. dulcis* is used to treat diabetes in India and hypertension in Taiwan [26]. In Brazil it has been used to treat various problems such as hemorrhoids and wounds [27], while it is used to manage sickle-cell disease in Nigeria [28] and applied to a wide range of problems in Nicaragua, including anemia, burns and headaches [29]. It is also used to protect the stomach from ulcers caused by indomethacin [30]. In the laboratory, extracts of the plant have been shown to have anti-hyperglycemic, [26] antimicrobial [31] and antioxidant properties [32] [33]. Scoparinol, an isolate of the plant, was shown to have analgesic, diuretic, and anti-inflammatory activity, as well [34]. Other active principles in the plant have been called scoparic acid, scopadulcic acid, scopadulciol, and scopadulin [35].

The present study was designed to determine the antibacterial and cytotoxic property as well as antioxidant effects of the methanolic extract of *S. dulcis* through *In vitro* analysis.

2. MATERIALS AND METHODS

2.1 Collection and Identification of Plant

The plant part was collected from Madhupur of Tangail forest region of Bangladesh. Taxonomist of National Herbarium Bangladesh, Dhaka, identified the plant and an accession number was submitted (35987).

2.2 Extraction

The methanolic extract of the plant was prepared according to the previously reported method [36]. The fresh leaves of *Scoparia dulcis* were cut into pieces, washed, and air-dried at room temperature (24±2℃) for about 10 days. The dried leaves were milled into a coarse powder. The powdered plant material (552.5 g) was mixed with methanol and the mixture kept for 2 days at room temperature with uninterrupted shaking. The extract was collected using Buckner funnel, where the methanolic mix of the powder was poured under vacuum suction. The filtrate contained the crude drug extract of methanol. The methanol was evaporated and a concentrated crude drug extract of *Scoparia dulcis* leaves was obtained and was preserved at 4℃ in an alpine tube awaiting further use. The yield of the dry methanolic extract was 13.96%.

2.3 Antioxidant Assay

DPPH scavenging assay: The DPPH scavenging activity of *S. dulcis* was carried out according the method by Liu and Zhao [37]. The reaction mixture contained 2 ml of 95% ethanol, 0.1 M DPPH and 2 ml of the methanolic leaf extract of *S. dulcis* (50, 75, 100, 200, 300 µg/ml). The solution was incubated at 25ºC for 15 min, and the absorbance of *S. dulcis* was determined at 517 nm. The antioxidant activity of *S. dulcis* extract was evaluated according to the following formula (eq. 1):

$$\text{Scavenging rate (\%)} = [1-A]/ A_0 \times 100 \qquad (1)$$

Where A is absorbance of *S. dulcis* extract and A_0 is the absorbance of negative control (DPPH solution). Similar concentration of ascorbic acid was used as positive control.

2.4 Cytotoxic Assay

In vitro Brine shrimp lethality bioassay [38] technique, using nauplii of *Artemia salina* was used for the determination of toxic property of *S. dulcis*. *Vincristin sulphate* was used as a positive control, for the comparison. Four milligrams of the extract was dissolved in DMSO to get a concentration of varying concentrations 100, 50, 25, 12.50 and 6.25 µg/ml. 10 brine shrimp nauplii were then placed in each vial and allowed to stand for 24 hour. The vials were observed using a magnifying glass and the number of survivors in each vial were counted. The percentage of mortality of the nauplii was calculated for each concentration and the 50% lethal concentration (LC_{50}) values were determined.

2.5 Antimicrobial Property Investigation

Antimicrobial Activity: Stock solution was prepared by dissolving 10 mg of the methanolic crude drug extract in methanol. The disk for drug dissolving was prepared using sterilized filter paper. Papers were punched uniformly to exactly 6 mm in diameter. Sample solutions of desired concentrations (100, 200, 400 and 500 µg/disk) were applied, using micropipette under an aseptic conditions. These disks were left for a

few minutes in aseptic condition for complete evaporation of the solvent. Kanamycin disk, K-30 disks containing 30 µg/disk, were used as a standard. The *In vitro* disk diffusion assay of antibacterial screening [39], was used to determine the susceptibility of the pathogenic microorganisms to the test compound applied.

Preparation of fresh culture of the pathogenic organisms: The nutrient agar medium was prepared and dispersed in a number of test tubes to prepare slants (5 ml in each test tube). This was done to prepare (Axenic) cultures from the supplied cultures [40]. The test tubes were sterilized at 121℃ temperature and a pressure of 15 lbs/sq inch for 15 minutes. After sterilization, they were kept in an inclined position for solidification and then incubated at 37.5℃. The test organisms were transferred to the agar slants from the supplied cultures using an inoculating loop in aseptic condition. The culture was kept at 4℃ or less for bacterial growth for 12 hour. Then incubated at 37℃ for 24 hours to assure the growth of test organisms. These fresh (Axenic) cultures were then used for the sensitivity test.

The test plates were prepared for the disc diffusion test of the test samples. Bacterial suspensions were transferred to the sterile petri dishes in an aseptic area. The petri dishes were rotated several times, first clockwise and then anticlockwise to assure homogenous distribution of the test organisms. The media was poured into petri dishes at a uniform depth of approximately 4 mm.

The medium was congealed to room temperature in laminar airflow unit, then refrigerated at (4℃) for 24 hours. The zones of inhibition of the different samples were measured and recorded [41].

2.6 TLC Analysis of the Fraction

Extracts were checked by thin layer chromatography (TLC) on analytical plates over silical gel. The solvent systems used was Chloroform: Methanol (9:1). In this case, the spots were visualized by exposure of the plates to UV lamp. Different bands were observed and corresponding R_f values were determined. The R_f value of each spot was calculated,

$$R_f = \frac{Distance\ traveled\ by\ solute}{Distance\ traveled\ by\ solvent} \quad (2)$$

3. RESULTS AND DISCUSSION

3.1 Antioxidant Assay

DPPH is a relatively stable free radical and the assay determined the ability of methanolic extract of *S. dulcis* to reduce DPPH free radicals to the corresponding hydrazine by converting the unpaired electrons to paired ones. Antioxidants can act by converting the unpaired electron to paired one. The dose-dependent inhibition of DPPH radicals (Fig. 1) indicates that the extract causes reduction of the DPPH radical in a stoichiometric manner [42-44] with an inhibitory concentration (IC_{50}) of 196.66µg/ml compared with 56.182 µg/ml for the standard control (Table 1). Thus, the extract possesses moderate antioxidative capacity, which can reduce the exacerbation of free radicals.

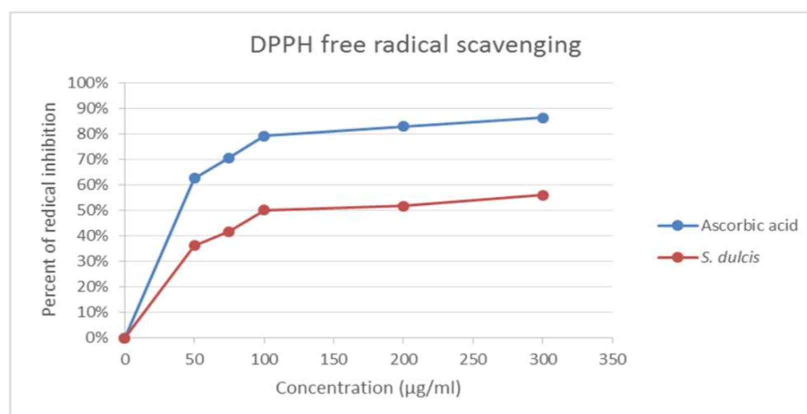

Fig. 1. Antioxidant property evaluation of methanolic extract of *S. dulcis*; from the graphical representation it is clear that our plant extract shows dose-dependent reduction of free radicals

A study on the hepatoprotective effect of *S. dulcis* on carbon tetrachloride induced acute liver injury in mice showed a significant inhibition of the increase of serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) [45,46]. A new tetracyclic diterpenoid, scopadulciol (6 beta-benzoyl-12-methyl-13-oxo-9(12) a, 9(12) b-dihomo-18-podocarpanol) isolated from the ethanol extract of *S. dulcis* mildly inhibited hog gastric H^+, K^+-ATPase [47].

Phytochemical studies shows that the plant contains flavonoids and phenolic compound [35]. Many plants contain sufficient amount of antioxidant compound including Vitamin C and E, carotenoids, flavonoids, tannins and they are useful in terms of scavenging the excess free radicals from the human body. Therefore, it may be possible that the flavonoids and phenolic compounds present in the methanolic extract of the *S. dulcis*, are responsible for the antioxidant activity.

3.2 Antimicrobial Assay

The zones of inhibition were observed against selective bacteria at a particular concentrations (Table 2). The methanolic extract of the leaves of *S. dulcis* showed higher activity against *E. coli*. At higher concentrations of 400 µg/disk and 500 µg/disk, the extract also showed good inhibitions against the other microorganisms. However, the extract showed negligible or no activity against the Gram-negative bacteria *S. dysenteriae* [31].

The findings of our present study and earlier reports of antibacterial activity suggest that the crude plant extract has pronounced potency against gram-positive than gram-negative bacteria. From this point of view, this extract may be indicative as broad-spectrum antibiotic [48,49].

3.3 Cytotoxic Assay

In cytotoxic test activity, percent of mortality increased gradually with the increase in concentration of the test samples. LC_{50} values obtained from the best-fit line slope (Fig. 3) were 29.868 µg/ml and 24.879 µg/ml for *S. dulcis* and vincristine sulphate, respectively.

Betulinic acid (a compound obtained from *S. dulcis*), and the ethanol extract of *S. dulcis* both showed anti-inflammatory activity against λ-carrageenan-induced paw edema in mice [50]. This compound also showed anticancer and cytotoxic property [51]. The diterpenes obtained from *S. dulcis* also possess cytotoxic potential [52,53].

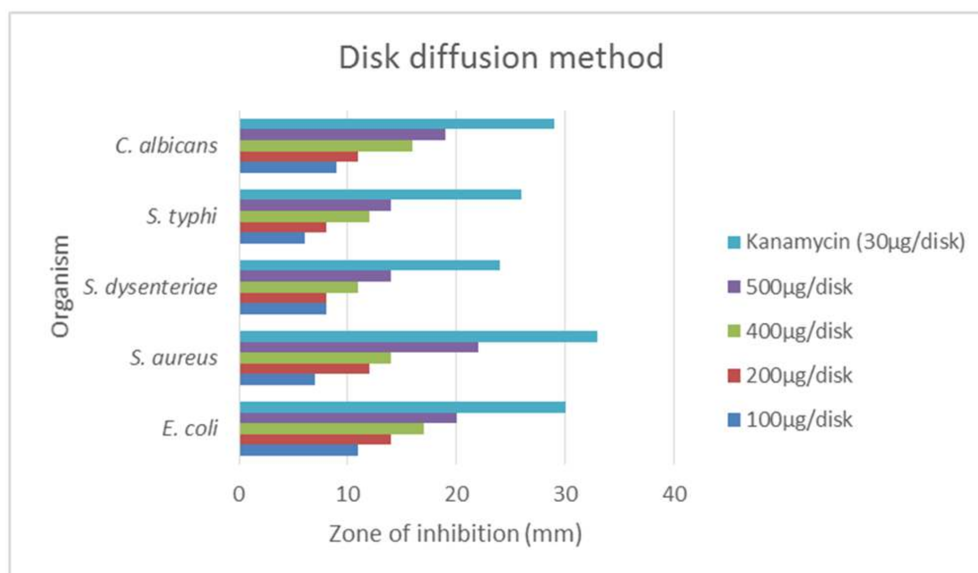

Fig. 2. Schematic presentation of bacterial growth inhibition; crude extract revealed its potent effectiveness at the concentration of 500 µg/disk; there were two gram-positive and three gram-negative microbes used; the extract was found to be similarly effective for both classes of organisms

Table 1. Absorbance recorded at different concentration of methanolic extract of *S. dulcis* and ascorbic acid

Group	Concentration (µg/ml)	Absorbance	IC_{50}
Ascorbic acid	50	0.445	56.182 µg/ml
	75	0.374	
	100	0.298	
	200	0.265	
	300	0.235	
S. dulcis	50	0.671	196.66 µg/ml
	75	0.615	
	100	0.562	
	200	0.536	
	300	0.507	

Table 2. Tabulation of zone of inhibition from agar media bacterial culture

Group	Concentration (µg/disk)	Microbial culture with zone of inhibition (mm)				
		E. coli	*S. aureus*	*S. dysenteriae*	*S. typhi*	*C. albican*
S. dulcis	100	11	7	8	6	9
	200	14	12	8	8	11
	400	17	14	11	12	16
	500	20	22	14	14	19
Kanamycin	30	30	33	24	26	29

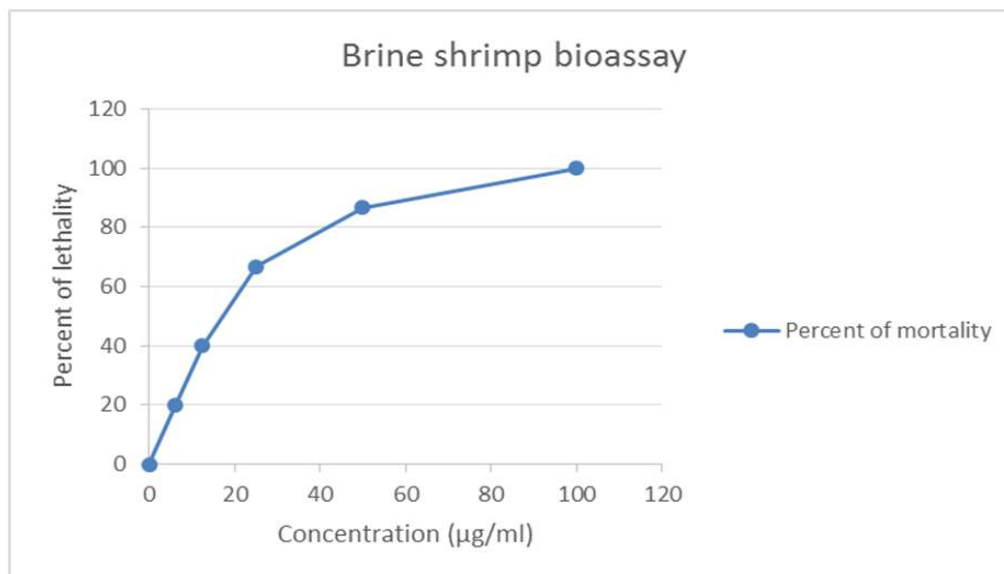

Fig. 3. Graphical representation of brine shrimp lethality bioassay; with the increase of extract concentration percentage of mortality increases

The brine shrimp lethality bioassay is very useful to assess the bioactivity of the plant extracts which in most cases correlate reasonably well with cytotoxic and anti-tumor properties [54]. LC_{50} values of *S. dulcis* revealed its considerable cytotoxic potency. Significant amounts of phenolics and flavonoids may be present and may be responsible for its promising cytotoxic activity [55,56] and the possible mechanism of cytotoxicity against brine shrimp nauplii may be due to poisonous effects on cell mitosis.

The brine shrimp lethality bioassay, using *Artemia salina*, is an inexpensive method to test

cytotoxicity. It is an indicator of antitumor and pesticidal activity [57] of a plant extract, as well as the antiviral, antiplasmodial, antimalarial, etc. [58] activities can be predicted. This present study with methanolic crude extract of *S. dulcis* reveals a prominent result over brine shrimp model. The observed LC_{50} (29.868 µg/ml) was very low, suggesting the existence of toxic compounds in its phytochemical library. The plant extract can be selected for further cell line analysis, to develop new pharmaceutical agents.

3.4 TLC Assay for Compound Detection

Observation of the TLC plates under a UV lamp resulted in the following results (Table 3).

Four nonpolar compounds were present with R_f values of 0.08, 0.23, 0.34 and 0.39. Four compounds were in between polar and nonpolar with R_f values of 0.46, 0.53, 0.63 and 0.69. Three non-chromophoric compounds with R_f values of 0.22 (nonpolar) and 0.62 (semi polar) and 0.69 (semi polar or polar). Thus, many compounds were present and isolation of the pure compounds is necessary.

Table 3. Different color spots observed in TLC plate and their estimated R_f value

Color of spot	R_f
Yellow brown	0.08
Light yellow	0.23
Yellow	0.34
Orange	0.39
Light yellow	0.46
Yellow	0.53
Yellow brown	0.63
Orange	0.69

4. CONCLUSION

This work has demonstrated that the methanolic extracts of *S. dulcis* leaves possesses promising antioxidant, cytotoxic and antimicrobial potentials, thereby lending support to the traditional use of the plant in the treatment of infectous and inflammatory disorders. However, further studies need to be carried out to understand the exact mechanisms of such actions and to isolate the active principles responsible for the observed activities.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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