Comparative Study of \textit{In vitro} Antioxidant, Antibacterial and Cytotoxic Activity of Two Bangladeshi Medicinal Plants- \textit{Luffa cylindrica} L. and \textit{Luffa acutangula}

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\section*{ABSTRACT}

\textit{Luffa cylindrica} L. (LUCY) and \textit{Luffa acutangula} (LUAC) are popular & consumed as vegetables and foods and in folklore medicine to treat various ailments in Bangladesh. The purpose of the present study was to investigate the antioxidant, antibacterial and cytotoxic activities of the n-hexane, chloroform and ethyl acetate extracts of leaves of LUCY & LUAC. Antioxidant and antibacterial activities were evaluated using the 2, 2-diphenyl-1-picrylhydrazyl (DPPH) assay and disc diffusion method, respectively. The plants were also assessed for cytotoxic activity by brine shrimp lethality assay. Antioxidant activity of different concentrations of plant extracts were found to increase in a concentration dependent manner. The most prominent activity was found with ethyl acetate extract of LUCY and n-hexane extract of LUAC with an IC$_{50}$ value of 50.32 & 50.95 µg/mL, respectively. All extracts/fractions showed low to moderate levels of anti-bacterial activity against most of the tested strains (zone of inhibition = 5-13 mm). All extracts/fractions displayed considerable general toxicity towards brine shrimps. The LC$_{50}$ values of the extracts/fractions were of 15.92 to 33.69 µg/mL compared to vincristine sulphate (LC$_{50}$ = 0.91 µg/mL). This is the first report for cytotoxic activity of LUCY and antibacterial activity of LUAC leaves extracts. On the basis of results obtained, it is suggested that both LUCY and LUAC leaves extracts may be a potential source of natural antioxidants, antimicrobial compounds and anticancer agents to be used in the treatment of various oxidative disorders, infectious diseases caused by resistant microorganisms and cancer, respectively.

\textbf{Key words:} \textit{Luffa cylindrica} L., \textit{Luffa acutangula}, DPPH, disc diffusion, brine shrimp lethality bioassay

\section*{INTRODUCTION}

The study of diseases and their treatment have been existing since the dawn of human civilization. Also for the treatment of a range of diseases, herbal drugs have been used since ancient times as medicines. Medicinal plants have played a key role in world health. The World Health Organisation (WHO) estimated that 80% of the population of developing countries relies on traditional medicines, mostly plant drugs, for their primary health care needs. In spite of the great advances observed in modern medicine in recent decades, plants still make an important contribution to health care.

A large proportion of the Bangladeshi population for their physical and psychological health needs depend upon traditional systems of medicine. Medicinal plants have become the focus of intense study in terms of conservation and as to whether their traditional uses are supported by actual pharmacological effects or merely based on folklore.\cite{1-2}

The luffa, loofah, or lufah are tropical and subtropical vines comprising the genus \textit{Luffa}, the only genus of the subtribe Luffinae. The fruit of at least two species, \textit{Luffa acutangula} and \textit{Luffa aegyptiaca} (\textit{Luffa cylindrica}), is grown, harvested before maturity, and eaten as a vegetable, popular in Asia and Africa. The plant \textit{Luffa cylindrica} L. belonging to family Cucurbitaceae is commonly called as Rajakoshaataki or Sponge gourd\cite{3} cultivated throughout the world and is distributed mainly in tropical to warm-temperate areas.\cite{4} The plant is reputed to have antitubercular and antiseptic properties.\cite{5-7} Antioxidant activity of the seed oil\cite{8} & antiinflammatory activity of the seed extract were reported.\cite{9} Extracts of fruits showed antioxidant,\cite{10} antibacterial and antifungal activity.\cite{10} On the other hand \textit{Luffa acutangula} also known as ridge gourd is an important member of the
family Cucurbitaceae grown in Bangladesh as a year-round vegetable. It is nutritionally rich in vitamin A, C and Fe\(^{[1]}\) and was found to contain carbohydrate, protein, fat and rich in Cu, Ni, Zn, Pb, Co, Cd, Fe, Cr, Ca and Na.\(^{[12]}\) LUAC has a considerable medicinal importance. The fresh fruits of it showed a certain antioxidant activity.\(^{[13]}\) Alcohol and chloroform extracts of fruits were shown to have antidiabetic activity.\(^{[14]}\) The methanolic fruit extract was reported for anticancer activity.\(^{[15]}\) Its abortifacient, antitumor, ribosome inactivating and immunomodulatory activities were reported earlier.\(^{[16-18]}\) Recently it has gained attention from the nutritionists due to the presence of antioxidant activity.\(^{[14]}\)

Literature reviews pointed out that no studies combining the antioxidant, antibacterial and cytotoxic activities of the leaves of LUCY & LUAC have so far been undertaken. Coupled with our continuous interest of pharmacological screening of Bangladeshi medicinal plants, in this study we aimed to investigate the antioxidant, antibacterial and cytotoxic activities of the n-hexane, chloroform and ethyl acetate extracts of leaves of LUCY & LUAC.

**MATERIALS AND METHODS**

**Drugs and chemicals**

DPPH (1, 1-diphenyl, 2-picryl hydrazyl), was obtained from Sigma chemical co. USA. Ascorbic acid was obtained from SD Fine chem. Ltd., Biosar, India. DMSO (dimethyl sulfoxide) was purchased from Merck, Germany. Kanamycin was collected from Square Pharmaceuticals Ltd., Bangladesh.

**Collection and Identification of the plant**

The fresh leaves of LUCY and LUAC were collected during the month of January 2010 from the area of Barguna, Bangladesh and identified by DR. M.A. Razzaque Shah, Tissue Culture Specialist, BRAC Plant Biotechnology Laboratory, Bangladesh. The fresh leaves of the plants were first washed with water to remove adhering dirt and then cut into small pieces, sun dried for 4 days. After complete drying, the entire portions were pulverized into a coarse powder with the help of a grinding machine and were stored in an airtight container for further use.

**Extraction and solvent-solvent partitioning of plant material**

The dried leaves were coarsely powdered from which about 50 gm powders of each plant were extracted with 3 times ethanol of their weight in a flat bottom glass container, through occasional shaking and stirring for 7 days. The extracts were then filtered through filter paper (Double Rings filter paper 102, 11.0 cm). The filtrates were concentrated at 50 °C under reduced pressure using vacuum pump rotary evaporator (STUART RF3022C, UK) to afford a greenish mass. The concentrated ethanol extract was made slurry with water. The slurry was taken in a separating funnel and few ml of n-hexane (40 ml) was added. The funnel was shaken vigorously and allowed to stand for few minutes. The n-hexane layer (upper layer) was collected. The process was repeated two times. The combined n-hexane extract was concentrated. After n-hexane extraction, chloroform (40 ml) was added to the aqueous solution and the mass was shaken vigorously in a separating funnel. Then the funnel was allowed to stand for few minutes for the complete separation of the layers. The organic (lower layer) layer was collected. The process was repeated two times. The aqueous layer left after chloroform extraction was again extracted two times with ethyl acetate.

**ANTIOXIDANT ACTIVITY TEST**

**DPPH radical scavenging activity**

**i) Qualitative analysis**

A suitably diluted stock solutions were spotted on pre-coated silica gel TLC plates and the plates were developed in solvent systems of different polarities (polar, medium polar and non-polar) to resolve polar and non-polar components of the extracts. The plates were dried at room temperature and were sprayed with 0.02% DPPH in methanol. Bleaching of DPPH by the resolved band was observed for 10 minutes and the color changes (yellow on purple background) were noted.\(^{[19]}\)

**ii) Quantitative analysis**

The free radical scavenging activities (antioxidant capacity) of the plant extracts on the stable radical 1, 1-diphenyl-2-picrylhydrazyl (DPPH) were estimated by the method of Brand-Williams et al.\(^{[20]}\) During this experiment the test samples n-hexane, chloroform and ethyl acetate extracts of both plants at different concentrations were mixed with 3.0 ml of DPPH methanol solution. The antioxidant potential was assayed from the bleaching of purple colored methanol solution of DPPH radical by the plant extracts as compared to that of ascorbic acid by UV spectrophotometer (UV–1501PC SHIMADZU, Japan) at 517 nm. Ascorbic acid was used as a positive control. Percent scavenging of the DPPH free radical was measured using the following equation-

$$\% \text{ DPPH radical scavenging} = \left[ 1 - \frac{(As)}{(Ac)} \right] \times 100$$

Here, Ac = absorbance of control, As = absorbance of sample solution.

Then % inhibitions were plotted against respective concentrations used and from the graph IC\(_{50}\) was calculated. The lower IC\(_{50}\) indicates higher radical scavenging activity and vice versa.
**IN VITRO ANTIBACTERIAL SCREENING**

Antibacterial activities of n-hexane, chloroform and ethyl acetate extracts of both plants were carried out by disc diffusion method.[21-22] In this method, measured amount of the test samples were dissolved in definite volumes of solvent to give solutions of known concentration. Then sterile filter paper discs (5 mm diameters) were impregnated with known test substances and dried. The dried discs were placed on plates (Petri dishes, 120 mm diameters) containing a suitable medium (nutrient agar) seeded with the test organisms. These plates are kept at low temperature (4 °C) for 24 hours to allow maximum diffusion. The dried discs absorb water from the agar medium and the material under test is dissolved. The test material diffuses from the discs to the surrounding medium according to the physical law that controls the diffusion of molecules through agar gel. There is a gradual change of the tested material concentration on the agar surrounding each disc. The plates are then kept in an incubator (37 °C) for 24 hours to allow the growth of microorganism. If the test material has antimicrobial activity, it will inhibit the growth of the microorganism, giving a clear, distinct zone called “Zone of Inhibition”. The antimicrobial activity of the test agent is determined in terms of millimeter by measuring the diameter of the zone of inhibition. The greater zone of inhibition indicates the greater activity of the test material against the test organism.

In our present study, the antibacterial activity of n-hexane, chloroform, and ethyl acetate fraction of both plants were investigated in comparison with standard kanamycin (30 μg/disc) against a number of pathogenic Gram-positive (Bacillus megaterium, B. subtilis, Staphylococcus aureus and Sarcina lutea) and eight Gram-negative (Salmonella paratyphi, S. typhi, Vibrio parahemolyticus, V. mimicus, Escherichia coli, Shigella dysenteriae, S. boydii and Pseudomonas aeruginosa) bacteria. The microorganisms were collected as pure cultures from the Institute of Nutrition and Food Science (INFS), University of Dhaka, Bangladesh. The sample solution of the material to be tested was prepared by dissolving a definite amount of material in appropriate solvent to attain a concentration of 50 mg/ml. 10 μl of such solution was applied on sterile disc (5 mm diameter, filter paper) and allowed to dry off the solvent in an aseptic hood. Thus, such discs contain 500 μg of crude extracts. To compare the activity with standard antibiotics, Kanamycin (30 μg/disc) was used.

**CYTOTOXICITY TEST**

**Brine shrimp Lethality Bioassay**

Brine shrimp lethality bioassay was used for probable cytotoxic activity.[23-25] The eggs of Brine Shrimp (*Artemia salina* Leach) was collected from local pet shops and hatched in a tank at a temperature around 37 °C with constant oxygen supply. Two days were allowed to hatch and mature the nauplii. Stock solution of the sample was prepared by dissolving required amount of extract in specific volume of pure dimethyl sulfoxide (DMSO). With the help of a pasteur pipette nauplii were exposed to different concentrations of the extracts.

**Preparation of test groups**

20 mg of sample was dissolved in 2 ml of DMSO to obtain a solution having concentration of 10 μg/ml. From that test solution different volumes were added to premarked glass vials or test tubes containing 5 ml of seawater and 10 shrimp nauplii, so as to make the final concentration of samples in the vials or test tubes 10 μg/ml, 20 μg/ml, 40 μg/ml, 60 μg/ml, 80 μg/ml. Vincristine Sulphate and DMSO were used as positive and negative control respectively.

**Counting of nauplii**

After 24 hours, the vials were inspected using a magnifying glass and the number of survived nauplii in each vial was counted. From this data, the percent (%) of lethality of the brine shrimp nauplii was calculated for each concentration. The median lethal concentration (LC50) and 95% confidence intervals were determined as the measure of toxicity of the extract or fractions.

**STATISTICAL ANALYSIS**

Lethality assays were evaluated by Finney computer statistical program[26] to determine the LC50 values and 95% confidence intervals.

**RESULTS**

**Antioxidant activity**

n-hexane, chloroform, ethyl acetate extracts for the leaves of both the plants showed excellent antioxidant activity with the IC50 value of 56.27 μg/ml, 61.24 μg/ml, 50.32 μg/ml respectively for LUCY and 50.95 μg/ml, 57.81 μg/ml respectively for LUAC compared with the standard ascorbic acid with IC 50 value of 43.22 μg/ml, 51.77 μg/ml, 50.95 μg/ml, 57.81 μg/ml, 51.77 μg/ml, 50.95 μg/ml. From that Table 2 shows the antioxidant activity of LUCY & LUAC with the zone of inhibition value ranged from 5-13 mm by disc diffusion method. Among different fractions tested, n-hexane extracts of both plants exhibited potent inhibitory
activity followed by chloroform extracts whereas ethyl acetate extracts showed little or no activity on the tested microorganisms. The most sensitivity was observed in *Staphylococcus aureus* (13 mm), *Bacillus megaterium* (13 mm) & *Shigella dysenteriae* (13 mm) by n-hexane extract of LUAC and in *Salmonella typhi* (12 mm) by the same extract of LUCY.

### DISCUSSION

Antioxidants act as a major defense against radical mediated toxicity by protecting the damages caused by free radicals. It is generally assumed that frequent consumption of plant-derived phytochemicals from vegetables, fruit, tea and herbs may contribute to shift the balance toward an adequate antioxidant status. Thus interest in natural antioxidant, especially of plant origin, has greatly increased in recent years.\[27\] In this study, antioxidant potential of n-hexane, chloroform and ethyl acetate extracts of both plants was evaluated based on the ability to scavenge the DPPH. This assay is highly important to provide information about the reactivity of organic compounds with stable free radicals,
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It could be assumed that isolation and purification of active constituents from these active extracts would lead to antioxidant compounds with comparable activity to that of the standard, Ascorbic acid. So it is assumed that the antioxidant activity of the plant extracts depends on: the type and the polarity of the extracting solvent, the extracting technique, the purity of the active principle, the antioxidant test and the substrate used.

It was observed that some phytochemicals such as alkaloids, flavonoids, glycosides, steroids and saponins were commonly present in all genus of Cucurbitaceae and some were found species specific. So it is assumed that the antioxidant potential may be due to the common presence of various secondary metabolites. The essence of these secondary metabolites is also beneficial for maintenance of human health and chronic degenerative diseases.

The zone of inhibition obtained of the n-hexane, chloroform & ethyl acetate extracts of LUCY and LUAC and the standard drug for the antibacterial activity are shown in Table 3 and found in the range of 5-13 mm against all tested bacteria. The n-hexane extracts of both the plants were found to have potent inhibitory activity against both gram positive and gram negative organisms followed by chloroform extract. This inhibitory activity may be attributed to be the presence of some active principles in them which are able to restrict the growth of bacteria. These active principles may inhibit protein synthesis of bacterial cell wall or alter the membrane function, inhibit protein synthesis or synthesis of purine and pyrimidines, hinder respiration or antagonize the metabolic pathways of microorganism leading to retardation of growth of bacteria. These active principles in these plants could be used as potent antibiotics.

Furthermore the ethyl acetate extracts of both the plants showed poor or no activity against tested organisms. The inhibitory activity to different microorganisms of LUCY supports with an earlier report in which the ethanolic extracts because of the odd number of electrons. DPPH shows a strong absorption band at 517 nm in visible spectrum (deep violet color). As the electron became paired in the presence of free radical scavenging, the absorption vanishes and the resulting discoloration stoichiometrically coincides with the number of electrons taken up. The bleaching of DPPH absorption is representative of the capacity of the test drugs to scavenge the free radicals independently. All extracts were found to exhibit antioxidant activity in the qualitative assay by displaying yellowish spots against purple background after the TLC plate being sprayed with the DPPH solution. In the quantitative assay the most promising antioxidant potential was observed with the ethyl acetate extract for LUCY and n-hexane extract for LUAC with an IC$_{50}$ value of 50.32 µg/mL and 50.95 µg/mL respectively compared to 43.22 µg/mL for ascorbic acid (Table 2). This indicates that the plant extracts are electron donors so they can react with free radicals, convert them into more stable products and terminate the radical chain reaction. This may be important in protecting cellular DNA, lipids and proteins from free radical damage. The percent inhibition of ethyl acetate extract at 100 µg/mL was about two fold less (41.53%) than that of chloroform extract in case of LUCY. The maximum inhibition or reduction of the DPPH absorbance with the chloroform extract for LUCY and ethyl acetate extract for LUAC was 83.23% and 80.63% respectively at the highest test concentration of 100 µg/mL. Standard ascorbic acid was found to have 86.53% activity at the concentration of 100 µg/mL. Our findings are in concordance with the previous studies of fruit & seed extracts of LUCY and fruit extracts of LUAC. Although the antioxidant activity of the extracts was considerably lower than the positive control, this is often the case with crude extracts. The positive control is a pure compound whereas the extracts are mixtures of several compounds. The compounds which were actually responsible for the antioxidant activities of the extracts were present in much lower concentrations than the concentrations of the crude extracts. Therefore, it could be assumed that isolation and purification of active constituents from these active extracts would lead to antioxidant compounds with comparable activity to that of the standard, Ascorbic acid. So it is assumed that the antioxidant activity of the plant extracts depends on: the type and the polarity of the extracting solvent, the extracting technique, the purity of the active principle, the antioxidant test and the substrate used.

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of LUCY fruits were found to have significant antibacterial activity [10]. Highest antibacterial activity was shown by n-hexane extract of LUAC against Staphylococcus aureus (13 mm), Bacillus megaterium (13 mm) & Shigella dysenteriae (13 mm) and of LUCY against Salmonella typhi (12 mm). The most resistant bacteria were Escherichia coli (5 mm), Pseudomonas aeruginosa (5 mm), Salmonella paratyphi (5 mm), Vibrio parahaemolyticus (5 mm) observed in ethyl acetate extracts of LUAC. The results obtained in the present study revealed that Gram positive bacteria were more sensitive than Gram negative bacteria to the plant extracts. Various workers have already reported similar results.[34-36] The difference in sensitivity might be ascribed to the difference in morphological constitutions between Gram-positive and Gram-negative organisms. Many plant species present inhibition zones of differing diameters; however, size difference of the inhibition zone depends primarily upon many factors for e.g. diffusion capacity of substances (present in the extracts) in the agar medium, antimicrobial activity of diffused substances, growth and metabolic activity of microorganisms in the medium. Inhibition zone diameter can further be associated with polarities of substances which make up the tested extracts and also with cell wall composition of test organisms since Gram-positive bacteria present cell walls with lower lipid levels than do Gram-negative bacteria.[37]

The brine shrimp lethality assay (BSL) has been used extensively in the primary screening of the crude extracts as well as the isolated compounds to evaluate the toxicity towards brine shrimps, which could also provide an indication of possible cytoxic properties of the test materials.[34] It has been established that the cytotoxic compounds generally exhibit significant activity in the BSL assay, and this assay can be recommended as a guide for the detection of antitumour and pesticidal compounds because of its simplicity and low cost.[38] Earlier reports in several plant extracts showed a good correlation of this bioassay with the cytotoxic activity.[39] The assay also exhibited a good correlation with cytotoxicity in cell lines such as 9KB, P388, L5178Y and L1210.[34][40-42] In the present study all extracts and fractions of LUCY & LUAC displayed considerable general toxicity & different mortality rate towards shrimp nauplii (Table 4). The mortality rate of nauplii was found to be increased in concentration of each of the samples. It was found that very low concentrations of plant extracts (10 μg/mL) were detrimental to the shrimp nauplii. The LC_{50} values of the plant extracts/fractions were within the range of 15.92 to 33.69 μg/mL, which was compared to that of the positive control (vincristine sulphate) with the value of 0.91 μg/mL (data not shown) determined earlier in our laboratory.[42] Ethyl acetate extract of LUCY and n-hexane extract of LUAC showed the highest level of toxicity with the LC_{50} values of 15.92 and 20.40 μg/mL, respectively. Previous studies of LUAC have already demonstrated the occurrence of cytotoxic activity,[43] although no similar observation was found in case of LUCY. The inhibitory effect of the extract might be due to the toxic compounds present in the active fraction that possess ovicidal and larvicidal properties. The metabolites either affected the embryonic development or slay the eggs.[44] So the cytotoxic effects of the plant extracts enunciate that it can be selected for further cell line assay because there is a correlation between cytotoxicity and activity against the brine shrimp nauplii using extracts.[44-45]

It was found that the antimicrobial activity of the extracts correlated strongly with the DPPH activity. This may be due to the availability of the antioxidative compounds to exert different inhibitory effect against tested organisms.[46] Moreover plant-derived extracts containing antioxidant principles showed cytotoxicity towards tumor cells[47] and antitumor activity in experimental animals.[48] Antitumor activity of these antioxidants is either through induction of apoptosis[49] or by inhibition of neovascularization.[50] The implication of free radicals in tumors is well documented.[51-52]

Finally it was observed from Table 1, Table 2, Table 3 and Table 4 that LUCY & LUAC extracts were different in their antioxidant, antibacterial as well as cytotoxic activities depending on the extractive solvents used. This result agrees favourably with the suggestion of Oloke and Kolawole[53] that bioactive components of any medicinal plant may differ in their solubility depending on the extractive solvents used. Takazawa et al.[34] suggested that there is a need to employ broad range of extractive solvents in the extraction of possible phytochemicals from medicinal plants. Besides the variation of activities of the plant extracts is presumed to be due to the presence of different active compounds and their degree of nature to different solvents.

**CONCLUSION**

From the experiment it has shown that different extracts (n-hexane, chloroform and ethyl acetate) have been used in vitro to inhibit the growth of some disease causing bacteria. It can therefore be suggested that plant extracts have great potential as antimicrobial compounds against microorganisms and they can be used in the treatment of infectious diseases caused by resistant microorganisms. They can also be a source of natural antioxidants & anticancer agents. The data obtained showed that n-hexane extract for LUAC and ethyl acetate extract for LUCY are more potent at all of the activities tested in the present study except in antibacterial activity where n-hexane fraction showed strongest activity for both LUCY and LUAC. Due to their antibacterial and antioxidant activities LUCY &
LUAC extracts have promising potential as a source of natural antioxidant and antimicrobial agents. Therefore these results are encouraging enough to pursue characterization of these fractions in different other models in detail. Further studies may also be conducted to isolate & purify the active constituents to evaluate the cytotoxic activity in human cell line cultures.

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