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# In-vitro Antioxidant and Cytotoxicity Studies of *Annona squamosa* Linn.

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**ABSTRACT**

n-Hexane, chloroform and methanol soluble extracts of the leaves of *Annona squamosa* were screened for their possible antioxidant activitiy by DPPH free radical scavenging and cytotoxicity by brine shrimp lethality bioassay. In DPPH radical scavenging assay, methanol soluble extract was found to be the most potent with an $IC_{50}$ value of 103.5 µg/ml. The amount of total phenolics was also found to the highest in the methanol soluble extract (283.16 ± 8.90 mg/g), followed by chloroform soluble extract (216.90 ± 4.48 mg/g). Here BHT and ascorbic acid were used as standards with $IC_{50}$ values 8.2 µg/ml and 25 µg/ml respectively. In the brine shrimp lethality bioassay, the most significant cytotoxicity was observed with chloroform soluble extract with an $LC_{50}$ of 4.16 µg/ml where vincristine sulphate was used as standard ($LC_{50}$ 0.29 µg/ml).

**Key Words**: *Annona squamosa,* DPPH, Folin-Ciocalteu Reagent, Phenolic Contents, Brine Shrimp

## INTRODUCTION

*Annona squamosa* Linn. (*Annonaceae*) locally known as sharifa, is a small tree, about 20 feet-high, with oblong to lanceolate leaves, greenish flowers and warty skinned segmented sweet fruits, planted as a fruit plant in different areas of Bangladesh (Ghani, 2003). Literatures of many research works prove that almost all parts of *A. squamosa* possesses medicinal property (Atique et al., 1985; Shirwaikar et al., 2004; Rathore et al., 1990; Ghani, 2003). Leaves contain anti-diabetic, spasmogenic, spasmolytic and oxytocic properties (Atique et al., 1985; Shirwaikar et al., 2004; Ghani, 2003). Ripe fruit is maturant and the mixture along with salt is used against malignant tumors to hasten suppuration (Rathore et al., 1990). Leaves bark and unripe fruits are used to treat diarrhoea and dysentery. Leaves and tender stems contain alkaloids, anonaine, roemerine, norcorydine, corydine, isocorydine and lanuginosine. Fruit pulp contains polyphenols, vitamin C and folic acid. Roots and root-barks contain alkaloids, diazepine, reticuline and squamolone (Ghani, 2003). Recently, interest has increased considerably in finding naturally occurring antioxidants in foods or medicinal materials to replace synthetic antioxidants, which are being restricted due to their side effects such as carcinogenicity. In addition, natural antioxidants have the capacity to improve food quality and stability and can also act as nutraceuticals to terminate free radical chain reactions in biological systems and thus may provide additional health benefits to consumers (Brand-Williams et al., 1995). Again brine shrimp lethality bioassay indicates cytotoxicity as well as a wide range of pharmacological activities such as antimicrobial, antiviral, pesticidal and anti-tumor etc. of the plant extracts (Meyer et al., 1982; McLughilin et al., 1998).In the present study, we evaluated the antioxidant and cytotoxic activity of leaf extracts of *A. squamosa.*

## MATERIALS AND METHODS

### *Plant Material*

The leaves of *A. squamosa* was collected from Dhaka in the month of July 2007. A voucher specimen for this collection has been maintained in Bangladesh National Herbarium, Dhaka, Bangladesh.

### *Extraction*

The powdered plant sample (300 g) was subjected to successive soxhlet extraction with the solvents with increasing order of polarity i.e. n-Hexane, Chloroform and Methanol. Subsequent

evaporation of solvents afforded 2.1 g of *n*-hexane soluble extract, 1.7 g of chloroform soluble extract and 3.7 g of methanol soluble extract.

**ANTIOXIDANT ACTIVITY**

***DPPH Radical Scavenging Activity***

The free radical scavenging activity of the extracts, based on the scavenging activity of the stable 1,1 diphenyl-2- picrylhydrazyl (DPPH) free radical, was determined by the method of Brand-Williams *et al.,* 1995. 2.0 ml of a methanol solution of the extract at different concentration were mixed with 3.0 ml of a DPPH methanol solution (20 µg/ml). Absorbance at 517nm was determined after 30 min, and the percentage inhibition activity was calculated from $[(A_0–A_1)/A_0]X100$, where $A_0$ is the absorbance of the control, and $A_1$ is the absorbance of the extract/ standard. BHT and ascorbic acid were the reagents used as standard.

***Assay for Total Phenolics***

The content of total phenols in extracts ware measured by a UV spectrophotemeter based on a colorimetric oxidation/reduction reaction (Majhenic *et al*., 2007). The oxidizing reagent used was Folin-Ciocalteu reagent. Gallic acid was used as standard. 2.5 ml of Folin-Ciocalteu reagent (diluted 10 times with water) and 2 ml of $Na_2CO_3$ (75 g/1L) were added to 0.5 ml of diluted extract (1 mg in 4 ml distilled water).The sample was incubated for 20 min at room temperature. For control sample, 0.5 ml distilled water was used. The absorbance was measured at 760 nm. These data were used to estimate the phenolic contents using a standard curve obtained from various concentration of gallic acid. The results were expressed as µg of gallic acid per mg of extract.

**Brine Shrimp Lethality Bioassay**

Brine shrimp lethality bioassay (McLaughlin *et al*., 1998; Meyer et al., 1982; Persoone, 1980) technique was applied for the determination of general toxic property of the plant extractives.

***Preparation of positive control group***

Vincristine sulphate was used as the positive control. Measured amount of vincristine sulphate was dissolved in DMSO to get an initial concentration of 20 µg/ml from which serial dilutions were made using DMSO to get 10 µg/ml, 5 µg/ml, 2.5µg/ml, 1.25 µg/ml, 0.625 µg/ml, 0.3125 µg/ml, 0.15625 µg/ml, 0.078125 µg/ml, 0.0390 µg/ml. Then the solutions were added to the premarked vials containing ten live brine shrimp nauplii in 5 ml simulated sea water.

***Preparation of negative control group***

100 µl of DMSO was added to each of three pre-marked glass vials containing 5 ml of simulated sea water and 10 shrimp nauplii. If the brine shrimps in these vials show a rapid mortality, then the test is considered as invalid as the nauplii died due to some reasons other than the cytotoxicity of the compounds.

***Preparation of test groups***

2 mg of each of the extracts was dissolved in DMSO and solutions of varying concentrations such as 200, 100, 50, 25, 12.50, 6.25, 3.125, 1.563, 0.781 and 0.39 µg / ml were obtained by serial dilution technique. Then the solutions were added to the premarked vials containing ten live brine shrimp nauplii in 5 ml simulated sea water.

***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 ($LC_{50}$) of the test samples was obtained by a plot of percentage of the shrimps killed against the logarithm of the sample concentration.

**RESULTS**

***The Amount of Phenolic Compounds***

The contents of total phenolics in the extracts of *A. squamosa* were determined using the Folin–Ciocalteu assay, calculated from regression equation of calibration curve ($y = 0.0162x + 0.0232$, $R^2 = 0.9985$) and were expressed as Gallic acid equivalents (Table -1).

**Table -1: Amount of total phenolic compounds in *A. Squamosa*.**

| Sample | Total phenols mg/g plant extract ( in Gallic acid equivalents) |
|---|---|
| n-Hexane soluble extract | 89.49 ± 2.48* |
| Chloroform soluble extract | 216.90 ± 4.48* |
| Methanol soluble extract | 283.16 ± 8.90* |

Results are mean ± SD of three parallel measurements.
Asterisks show the statistical differences of the values at $p<0.001$ probability level.

***DPPH radical scavenging activity***

The extracts were able to reduce the stable radical DPPH to the yellow-coloured diphenylpicrylhydrazine. The $IC_{50}$ values of the *n*-hexane soluble extract, chloroform soluble extract and methanol soluble extract were 170.5 µg/ml, 127 µg/ml and 103.5 µg/ml. The $IC_{50}$ values of the positive control ascorbic acid and BHT was 8.2 and 25 µg/ml.

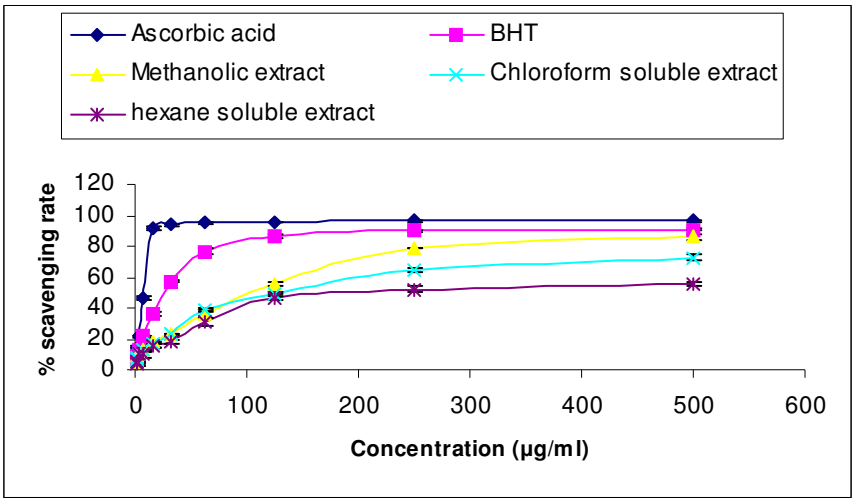


**Figure1:** Scavenging activity of different extracts of *A. squamosa* against 1,1-diphenyl-2-picrylhydrazyl radical. Results are mean ± SD of three parallel measurements.

***Brine Shrimp Lethality Bioassay***

**Table 2: Results of the test samples of *A. squamosa***

| Sample | $LC_{50}$ (µg/ml) | Regression Equation | $R^2$ |
|---|---|---|---|
| **Vincristine Sulfate (Positive control)** | 0.29 | y = 28.523x + 65.204 | 0.9269 |
| **n-Hexane soluble extract** | 42.65 | y = 20.202x + 17.131 | 0.9872 |
| **Chloroform soluble extract** | 4.16 | y = 26.578x + 33.511 | 0.9504 |
| **Methanol soluble extract** | 14.12 | y = 24.363x + 21.94 | 0.9689 |

The toxicity of *n*-hexane soluble extract (HSE), chloroform soluble extract (CSE) and methanol soluble extract (MSE) to brine shrimp was evaluated on *A. salina*. Table 2 shows the results of the brine shrimp lethality test after 24 hours of exposure to the samples and the positive control,

vincristine sulphate (VS). The $LC_{50}$ values of *n*-hexane soluble extract, chloroform soluble extract and methanol soluble extract were 42.65 µg/ml, 4.16 µg/ml and 14.12 µg/ml, respectively.

**DISCUSSION**

DPPH is a stable free radical and accepts an electron or hydrogen radical to become a stable diamagnetic molecule (Soares, Dins, Cunha, & Ameida, 1997). DPPH is usually used as a reagent to evaluate free radical scavenging activity of antioxidants (Oyaizu, 1986). The experimental data reveal that all these extracts are likely to have the effect of scavenging free radical. From Figure 1 it was observed that a dose–response relationship is found in the DPPH radical scavenging activity; the activity increased as the concentration increased for each extract. The key role of phenolic compounds as scavangers of free radical is emphasized in several reports (Moller *et al.,* 1999; Madsen *et al.,* 1996). Therefore, it is worthwhile to determine their total amount in the plants chosen for the study. It can be observed that the content of phenolics in the extracts correlates with the antioxidant activity, being highest in methanol soluble extract (283.16 ± 8.90 µg/mg GAE) and lowest in Hexane soluble extract (89.49 ± 2.48 µg/mg GAE). The involvement of free radicals, especially their increased production, appears to be a feature of most, if not all human diseases, including cardiovascular disease and cancer (Deighton et al., 2000). It has been found that cysteine, glutathione, ascorbic acid, tocopherol, flavonoids, tannins, and aromatic amines (p-phenylene diamine, p-aminophenol, etc.), reduce and decolourise DPPH by their hydrogen donating ability (Blois, 1958; Yokozawa et al., 1998). Phenolic compounds of the plant extracts are probably involved in their antiradical activity. From the results of the brine shrimp lethality bioassay, it was found that all the fractions possess cytotoxic principles and have considerable cytotoxic potency. Comparison with positive control (vincristine sulphate) signifies that cytotoxicities exhibited by the chloroform soluble extract and methanol soluble extract are promising and further bioactivity-guided investigation can be done to find out potent antitumor and pesticidal compounds.

**CONCLUSION**

The bioactivities exhibited by the different extractives demand further studies for the isolation and identification of individual bioactive compounds and also *in vivo* studies are needed for understanding their mechanism of actions better.

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