THIN LAYER CHROMATOGRAPHIC STUDIES AND *IN VITRO* FREE RADICAL SCAVENGING ACTIVITY OF *ANNONA SQUAMOSA* LEAF EXTRACTS

Amartya Bose[1], Sanjib Bhattacharya[2*], J. N. Pandey[1], Moulisha Biswas[1]

[1]Bengal Institute of Pharmaceutical Sciences, Kalyani, Nadia 741235, West Bengal, India

[2]Bengal School of Technology, Delhi Road, Sugandha, Hooghly 712102, West Bengal, India

***For correspondence:** E-mail: sakkwai@yahoo.com

Summary

Annona squamosa L. (Annonaceae), known as custard apple, is commonly found in deciduous forests and also cultivated in wild in various parts of India**.** The present study assessed the different solvent extracts of *A. squamosa* leaf for thin layer chromatography (TLC) and also evaluated their *in vitro* free radical scavenging potential by 1,1-diphenyl-2-picryl-hydrazyl (DPPH) radical scavenging assay. The chloroform extract yielded maximum spots in TLC, followed by petroleum ether and methanol extracts respectively. All of the extracts exhibited potent *in vitro* free radical scavenging activity that increased with extract concentration. The methanol extract was found to be the most potent in this regard.

Key words: Free radical scavenging, leaf, antioxidant, *Annona squamosa.*

Introduction

Antioxidants protect living organisms from damage caused by uncontrolled production of reactive oxygen species (ROS) and the concomitant lipid peroxidation, protein damage and DNA strand breaking. Current interest is focused on the potential role of antioxidants and antioxidant enzymes in the treatment and prevention of atherosclerosis, heart failure, neurodegenerative disorders, aging, cancer, diabetes mellitus and several others diseases (1).

Antioxidants are added to a variety of foods to prevent free radical induced lipid peroxidation, which is responsible for the development of off-flavors and the undesirable chemical compounds in food (2). These ROS cause destructive and irreversible damage to the components of a cell, such as lipids, proteins DNA and other macromolecules. Although normal cells possess antioxidant defense systems against ROS in the cells induces diseases such as cancer and aging (3).

ROS are formed and degraded by all aerobic organisms. ROS can readily react with most biomolecules including proteins, lipids, lipoproteins and DNA. Exogenous chemical and endogenous metabolic processes in the human body or in the food system might produce highly reactive oxygen species, which are capable of oxidizing biological molecules, resulting in tissue damage and cell death. When the mechanism of antioxidant protection becomes unbalanced by exogenous and endogenous factors, it results in inflammation, diabetes, genotoxicity, cancer and accelerating aging (4).

Antioxidant supplements or foods containing antioxidants may be used to help the human body reduce oxidative damage. The most commonly used antioxidants are BHA, BHT, propyl gallate and tert-butyl-hydroquinone (5). However, they have been suspected of being responsible for liver damage and carcinogenesis in laboratory animals. Therefore, the development and use of more effective antioxidants is desired.

Traditional medicine worldwide is being reevaluated by extensive research on different plant species and their therapeutic principles. Plants produce antioxidants to control the oxidative stress caused by sunbeams and oxygen, they can represent a source of new compounds with antioxidant activity.

Annona squamosa L. (Annonaceae), known as custard apple, is a small, semi-(or late) deciduous, much branched shrub or small tree, commonly found in deciduous forests and also cultivated in wild in various parts of India. It is a native of West Indies, now cultivated throughout India and other tropical countries. Literatures of many research works prove that every parts of *A. squamosa* possess medicinal property (6). Roots are employed internally in depression of spirits and spinal diseases. Bark is known to be a powerful astringent. In Ayurveda, the traditional system of Indian medicine, its fruits are considered as a good tonic, enrich blood, used as expectorant, increases muscular strength, cooling, lessens burning sensation and tendency to biliousness, sedative to heart and relieves vomiting (7-9). Due to uniqueness of leaf property in curing of different ailments, plant's this part was selected for the present chromatographic and antiradical study.

Materials and methods

Plant material: The mature leaves of *Annona squamosa* L. (Annonaceae) were collected during November 2010 from Nadia, West Bengal, India. The plant material was taxonomically identified at the Central National Herbarium, Botanical Survey of India, Howrah, West Bengal, India. The voucher specimen [CNH/I-I/(80)/2010/Tech.II/349] was maintained in our research laboratory for future reference. The plant material was shade-dried with occasional shifting and then powdered with mechanical grinder, passing through sieve no. 40, and stored in an air-tight container.

Preparation of plant extracts: The dried powdered material (350 g) was defatted with petroleum ether (60-80 °C), the percentage extractive value was 1 % w/w. The defatted powder material thus obtained was further extracted with chloroform and methanol for 72 h in a percolator. The solvent was distilled off in reduced pressure and resulting semisolid mass was vacuum dried using rotary flash evaporator to yield a solid residue and the percentage extractive values were accordingly 12.5 % w/w and 15 % w/w respectively. The preliminary phytochemical analysis was performed for all three extracts to identify the phytoconstituents present in the extracts (10).

Chemicals: L ascorbic acid (vitamin C) and 1,1-diphenyl-2-picryl-hydrazyl (DPPH) from Sigma Chemical Co. Ltd. (St. Louis, MO, USA). All other chemicals and reagents were of analytical grade obtained commercially.

Thin layer chromatographic studies: Each solvent extract was subjected to thin layer chromatography (TLC) as per standard one dimensional ascending method. The results and chromatograms are depicted in Figures 1-3.

Free radical scavenging activity measured by 1, 1-diphenyl-2-picryl-hydrazil: The free radical scavenging activity of all of the extracts were measured by 1,1-diphenyl-2-picryl-hydrazil (DPPH) using the reported method (11). Briefly, an 0.1 mM solution of DPPH in methanol was prepared, and 1 ml of this solution was added to 3 ml of the all of the extracts solution respectively in petroleum ether, chloroform and methanol at different concentrations (2, 4, 6, 8, 10, 15 μg/ml). The mixture were shaken vigorously and allowed to stand at room temperature for 30 min. Then the absorbance was measured at 517 nm using a UV-Vis spectrophotometer (Genesys 10 UV: Thermo Electron Corporation). Lower absorbance values of reaction mixture indicate higher free radical scavenging activity. The capability to scavenge the DPPH radical was calculated by using the following equation:

$$\text{DPPH scavenging effect (\%)} = [(A_0 - A_1) / A_0) \times 100]$$

Where, A_0 is the absorbance of the control reaction, and A_1 is the absorbance of presence of all of the extract samples and standard. The results are stated in Table 1 and Figure 4.

Results and discussion

Preliminary phytochemical studies showed the presence of terpenoids in the petroleum ether and chloroform extracts, whereas glycosides and carbohydrates in the methanol extract form *A. squamosa* leaf.

Among the various methods for separating plant constituents, the chromatographic procedure is the one of the most commonly used techniques of general application (12). Thin layer chromatography (TLC) involves the separation of mixtures of organic compounds on thin layers of adsorbents that are usually coated on glass, plastic, or aluminium sheets; and this particular technique is the easiest, cheapest and most widely used method for the characterization of natural products and their preparations (13).

The present thin layer chromatographic studies revealed the presence of maximum constituents in the chloroform extract, as it exhibited maximum numbers of well resolved spots. Despite showing maximum yield, the TLC profile of methanol extract was not much encouraging. The petroleum ether extract, on the other hand, despite showing very low yield

exhibited quite interesting TLC profile, although some spots were not properly resolved. All of these TLC profiles may serve as characteristic fingerprint of *A. squamosa* leaf. It would therefore be suitable for monitoring the identity and purity of the plant material and for detecting adulterations and substitutions.

The stable DPPH radical model is a widely used, relatively quick and precise method for the evaluation of free radical scavenging activity. The effects of antioxidants on DPPH radical scavenging is thought to be due to their hydrogen-donating ability. DPPH is a stable free radical that accepts an electron or hydrogen radical to become a stable diamagnetic molecule. The absorption maximum of a stable DPPH radical in methanol was 517 nm. The decrease in absorbance of DPPH radical caused by antioxidants, because of the reaction between antioxidant molecules and radical progressed, results in the scavenging of the radicals by hydrogen donation. It is visually noticeable that as a change in color from purple to yellow. Hence, DPPH is usually used as a substrate to evaluate the antioxidant activity of antioxidants (14). It has been reported that oxidative stress, which occurs when free radical formation exceeds the body's ability to protect itself, forms the biological basis of chronic conditions such as arteriosclerosis (15).

Based on the data obtained from the present study, all the extracts were effective free radical inhibitor or scavenger, that reacts with free radicals, which may limit free radical damage occurring in the human body. Figure 1 illustrates a significant decrease in the concentration of DPPH radicals due to the scavenging ability of the extracts and the reference compound. Free radical scavenging activity also increased with increasing concentration in the range of 2-15 µg/ml. The methanol extract was found to be the most potent comparable to that of ascorbic acid at the test concentration. The present preliminary study confirms marked *in vitro* free radical scavenging activity of *A. squamosa* leaf which may be due to presence of multitude of constituents as revealed by TLC.

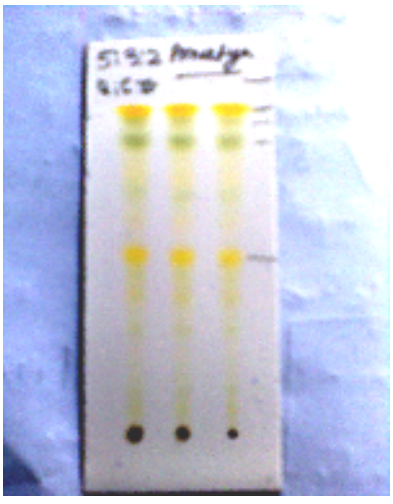

Fig. 1. TLC study of the pet. ether extract of *A. squamosai* leaf. Solvent system: benzene: chloroform: ethyl acetate (5: 3: 2). R_f values: 0.48, 0.79, 0.85, 0.90.

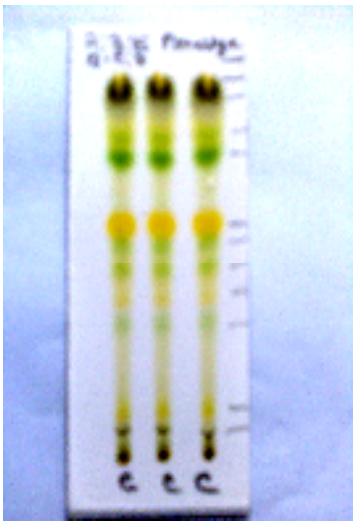

Fig. 2. TLC study of the chloroform extract of *A. squamosai* leaf. Solvent system: benzene: chloroform: ethyl acetate (2: 3: 5). R_f values: 0.03, 0.13, 0.33, 0.48, 0.55, 0.60, 0.76, 0.83, 0.92, 0.97.

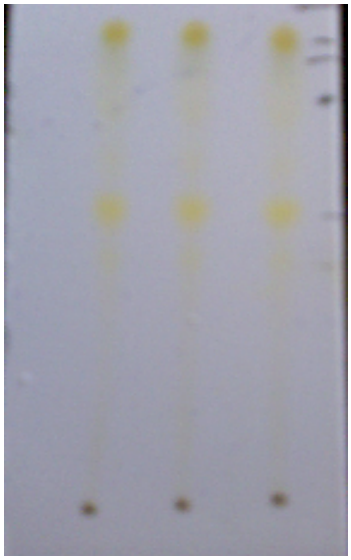

Fig. 3. TLC study of the methanol extract of *A. squamosai* leaf. Solvent system: chloroform: methanol (6: 4). R_f values: 0.23, 0.29, 0.35, 0.38.

Table 1. DPPH scavenging power of the different extracts of *A. squamosa* and vitamin C.

Extracts	Concentrations (µg/ml)	% of DPPH scavenging activity respectively	Mean ± SEM
Annona squamosa (Petroleum ether)	2, 4, 6, 8, 10, 15	27.29, 33.07, 38.99, 59.51, 69.89, 83.67	49.59±8.681
Annona squamosa (Chloroform)	2, 4, 6, 8, 10, 15	29.51, 37.65, 49.78, 58.92, 68.87, 80.55	54.76±7.73
Annona squamosa (Methanol)	2, 4, 6, 8, 10, 15	45.59, 59.29, 70.35, 79.92, 87.79, 96.83	73.77±7.57
Vitamin C	2, 4, 6, 8, 10, 15	25.70, 32.10, 42.50, 92.20, 97.23	57.946±15.29

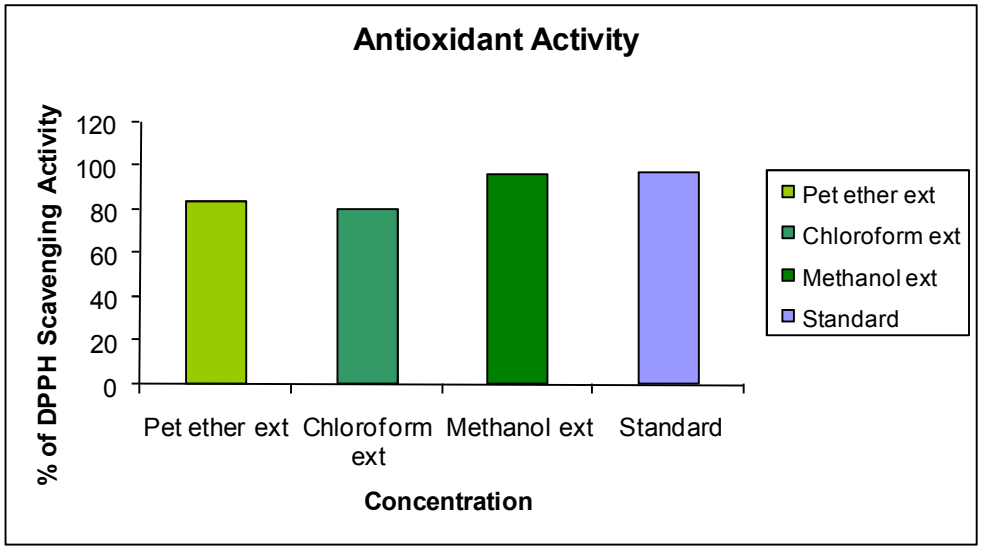

Fig. 4. DPPH scavenging effect of the different extracts of *A. squamosa* and vitamin C.

Acknowledgement

The authors are thankful to Mr. Subir K. Pal, President & CEO of Bengal Institute of Pharmaceutical Sciences for his kind co-operation and financial support to complete this work successfully.

References

1. Ajitha M, Rajnarayana K. Role of oxygen free radicals in human disease. Indian Drugs. 2001; 38: 545-554.
2. Halliwell B, Aeschbach R, Loliger J, Aruom OI. The characterization of antioxidants. Food Chem Toxicol. 1995; 33: 601-617.
3. Mates JM, Sanchez- Jimenez FM. Role of reactive oxygen species in apoptosis: Implications for cancer therapy. Int. J. Biochem. Cell Biol. 2000; 32: 57-170.
4. Buyukokurogla ME, Gulein L, Oktav M, Kufrevioglu OI. *In vitro* antioxidant properties of dantrolene sodium. Pharmacol Res. 2001; 44: 491-495.
5. Gulcin I, Beydemir S, Ahmet AH, Mahfuz E, Emin BM. *In vitro* antioxidant properties of morphine. Pharmacol Res. 2004; 49: 59-66.
6. Kirtikar, K.R. and Basu, B.D. (1968). Indian medicinal plants (vols. I and II). Lalit Mohan Basu, Allahabad, India.
7. Atique A, Iqbal M, Ghouse AKM, Use of *Annona squamosa* and *Piper nigrum* against diabetes, Fitoterapia, 56(3), 1985, 190-192.
8. Rathore DS, Custard apples, In: T.K. Bose. and S.K. Mitra (eds.). Fruits, tropical and subtropical. Naya Prokash. Calcutta, 1990, 449-468.
9. Ranjan, V, Exotic medicinal plants of district Lalitpur, Uttar Pradesh. Bulletin of Medico-Ethno Botanical Research, 20 (1-4), 1999, 54-65.
10. Kokate CK (1994): Practical Pharmacognosy. 4th Edition. New Delhi. Vallabh Prakashan. pp. 107-112.
11. Blois MS (1958): Antioxidant determinations by the use of stable free radical. Nature 26: 1199-1200.
12. Kokate CK, Purohit AP, Gokhale SB. Pharmacognosy. 34th ed. Nirali Prakashan, Pune, 2006, 114.
13. Stahl E. Apparatus and General Techniques. In: Stahl E, editor. Thin Layer Chromatography, A Laboratory Handbook. 2nd ed. Springer-Verlag, Berlin, Heidelberg, New York,1969, 52-85.
14. Chang LW. Yen WJ. Huang SC. Duh PD (2002): Antioxidant activity of sesame coat. Food Chem *78*: 347-354.
15. Fatimah ZI, Zaiton Z, Zamaludin M, Gaptor MT, Nafeeza ML, Khairul O. Effect on estrogen and palm vitamin F on malonaldehyde levels toward the development of arteriosclerosis in the New Zealand white rabbit. In : Packer L Ong SH. Eds. Biological Oxidants and antioxidants: Molecular Mechanism and Health Effects. Champaign. IL. AOCS Press.1998; 22.