ISSN: 2279 - 0594

Available Online at www.jbpr.in

Journal of Biomedical and Pharmaceutical Research 3 (2) 2014, 29-36

RESEARCH ARTICLE

CYTOTOXICITY AND PHYTOCHEMICAL STUDIES OF PUMPKIN SEED (*CUCURBITA MAXIMA* LINN.) EXTRACT.

Shahangir Biswas[1], Ahsan Habib[1], M. Manirujjaman[1,2], Belal Uddin[1], M.M.H. Khan[1], Sohel Hasan[1], Meftah Uddin[1], Minarul Islam[1], M.Khatun[1], M. A. Islam[1], Matiar Rahman[1]*

[1]Department of Biochemistry and Molecular Biology, University of Rajshahi, Bangladesh

[2]Department of Biochemistry, Gono Bishwabidyalay(Gono University) Savar, Dhaka, Bangladesh

Received 19 March 2014; Accepted 30 March 2014

ABSTRACT

Pumpkin *(Curcurbita maxima*) is a popular vegetable in our country. This plant is locally known as "Mistikumra".Pumpkin (Cucurbita sp.) seeds are a key food source for humans because they are a very good source of proteins (24–36.5%) and oil (31.5–51%)Pumpkin seeds have long been valued as an important natural food for men's health. Pumpkin seed extract is useful for immunomodulation, reproductive health and therapeutic advantage over a wide range of disease conditions. The intake of a whole extract of pumpkin seeds has been correlated with reduced benign prostate hyperplasia-associated symptoms. The antioxidative property of pumpkin seed extract could also improve fertility, and it helps to prevent arteriosclerosis, high blood pressure and heart diseases; it also stimulates metabolism of accumulated fats.It has potential for allergic reactions to pumpkin that is side effect of pumpkin seeds. Cytotoxic effect of Pumpkin *(Curcurbita maxima*) seed was studied against brine shrimp nauplii. In brine shrimp lethality bioassay, the LD_{50} value of methanol extracts and petroleum ether fraction of Pumpkin *(Curcurbita maxima*) seed were determined and found to be 31.70 ppm and 21.95 ppm respectively. As the petroleum ether fraction showed greater cytotoxic effect we made further study with this fraction. The petroleum ether fraction was subjected to thin layer chromatography on slica gel that yielded four fractions designated as PE-1, PE-2, PE-3, and PE-4, among them only PE-4 was suitable for further purification because it gave single band on two dimensional TLC. The PE-4 may be steroid because it showed pink colour after spraying vanillin sulfuric acid. The IR data revealed that the isolated PE-4 compound contained aromatic ring and ketonic groups (-CO-). NMR and Mass spectral analysis is further needed to elucidate the molecular structure of the compound. The experiments showed that it has a cytotoxic effect and IR data proved that it is steroid compound.

Key words: *Curcurbita maxima*, cytotoxic , Mistikumra, Chromatography, Bangladesh

INTRODUCTION:

Bangladesh is a developing and over populated country where the most of the people are living below the poverty level especially in rural area. Most of the people in Bangladesh are habituated with the local and indigenous food. Vegetables are very common in their everyday food menu and play an important role in the food chain. More than sixty, indigenous and exotic, vegetables are grown in Bangladesh, most of which are very cheap, available and popular to people for daily food intake. The enriched food value as well as medicinal property are still unexplored and may play a pivotal role in the development of potential new drugs for chemotherapy which might help to overcome the growing problem of resistance and also the toxicity of the currently available commercial antibiotics. The traditional medicinal methods, especially the use of vegetable may play a vital role to cover the basic health needs in the developing countries like Bangladesh [1].

Pumpkin seed is considered to be safe in when taken in food amounts and possibly safe when taken in amounts that produce medicinal effects. Human studies with pumpkin have reported few side effects. Also, there is the potential for allergic reactions to pumpkin. Medicinal amounts of pumpkin seed should not be taken during pregnancy or lactation .The seeds and seed extracts can cause allergic reactions. Antinutrients have been described in the seeds and leaves, including oxalates,

*Corresponding author: Professor Dr. Matiar Rahman | Email: matiar_rahman@yahoo.com

tannins, and cyanide. There are no known side-effects or contraindications for taking pumpkin seeds; however, we can make sure to consult with a health care[1].

Brine shrimp lethality bioassay is a recently developed procedure in the bioassay of the bioactive compounds. Natural product extracts and pure compounds can be tested for their bioactivity by this method. Here in vivo lethality, a simple zoological organism (Brine shrimp nauplii) is used as a convenient monitor for screening and fractionation in the discovery of new bioactive natural products. Generally, the median effective dose (ED50) values for cytotoxicities are one tenth of median lethal dose, LD_{50} values in the brine shrimp test.. The bioassay indicates cytotoxicity as well as a wide range of pharmacological activities (e.g. anticancer, antiviral, pesticidal, AIDS, etc) of the compound [3-6].

The brine shrimp lethality bioassay (BSLB) 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 cytotoxic properties of the test materials compound [7]. Because BLSB is the simple method useful for screening large number of extracts in the drug discovery process. The method allows the use of smaller quantity of the extracts and permits larger number of samples and dilutions within shorter time than using the original test vials compound[8].

This bioassay has also a good correlation with the human solid tumour cell lines. 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. Therefore 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 compound[9].

The *in vitro* cytotoxicity displayed by the plant extracts tested is an initial indicator of*in vivo* antitumour activity. However since a wide range of phytocompounds are capable of exhibiting nonspecific cytotoxicity, plant extracts with significant cytotoxic activity should be further assayed using animal models to confirm antitumour activity, and/or a battery of various cell lines to detect specific cytotoxicity. This step is necessary to eliminate cytotoxic compounds with little value for further investigation as anticancer agent compound [10].

In nature many plants and plants seed provided source of medicine at the earlier times. Plants have proven to be the most useful in curing diseases and provide an important source of pharma and medicine. Plants have great significance to the health of individuals. The medicinal importance of these plants lies in some chemical substances that produce a distinct physiological action on the body of human.

Phytochemical is nonnutritive chemical constituents of plants which occur naturally in it i.e termed as Phytochemical, or the chemical which is derived from plants are called Phytochemical. The herbal products today symbolize shelter in contrast to the synthetics that are regarded as unsafe to human and environment. Although herbs had been used for their medicinal, flavoring and aromatic character, over three quarters of the world population believes mainly on plants and plant extracts for health trouble. As population is increasing to a higher side day by day, there are not enough supply of drugs, there are excessive cost of treatments or curing, side effects of several allopathic drugs and development of resistance to at present used drugs for infectious diseases have led to increased importance on the use of plant materials as a source of medicines for a wide variety of human ailments compound [11].

MATERIALS AND METHODS:

Collection of pumpkin seed:

Pumpkin seeds are collected from local market. The seeds were cleaned, air dried, packed in polyethylene bag, sealed and stored at 4ºC for use in subsequent experiments.

Preparation of brine water:

38 g of sea-salt (non-ionized NaCl) was dissolved in one liter of sterilized distilled water and then filtered off to get clear solution. The P^H of the seawater was maintained between 8 and 9 using $NaHCO_3$ solution.

Hatching of brine shrimp eggs:

Brine shrimp eggs were collected from a fish shop of Kawran Bazar, Dhaka. Sea water was taken in the small tank and the shrimp eggs (1.5 g/L) were added to one side of the tank and this side was covered. The eggs were allowed for two days to hatch and mature as Nauplii (Larvae). Constant oxygen supply was carried out during the hatching time. The hatched shrimps were attracted to the lamp on the other side of the divided tank through the perforated dam. These nauplii were taken for this bioassay.

Preparation of the test sample:

Crude methanol extract (M_3) and petroleum ether fraction (3 mg of sea water each) were dissolved in 0.6 ml (600 µl) DMSO to get a concentration of 5 mg/ml for each of the sample. These samples were used as stock

solution. Five doses (10, 20, 40, 80 and 100 µg) of each sample were used for the lethality test of brine shrimp nauplii. With the help of a micropipette 10, 20, 40, 80 and 100 µl of each sample were transferred from the stock solution in 5 different vials. Sea water (brine water) was added to each vial making the volume up to 5 ml. The final concentration of the samples in these vials become 10, 20, 40, 80 and 100 µg/ml respectively. For each concentration control experiment was done. The experiment was repeated three times.

Preparation of the positive control group:

In the present study, ampicillin trihydrate was used as the positive control. 3 mg of ampicillin trihydrate was dissolved in 0.6 ml (600 µl) of DMSO to get a concentration of 5 mg/ml. This was used as stock solution of ampicillin trihydrate. With the help of a micropipette 10, 20, 40, 80 and 100 µl of the stock solution were transferred in 5 different vials. NaCl solution (brine water) was added to each vial making the volume up to 5 ml. The final concentration of ampicillin trihydrate in the vials became 10, 20, 40, 80 and 100 µg/ml respectively. The experiment was repeated three times.

Application of brine shrimp nauplii:

10 living nauplii were transferred to each of the vials. A magnifying glass was used for convenient counting of the nauplii. If the counting of 10 nauplii was not being possible accurately, then a variation in counting from 9-11 might be allowed.

Counting of nauplii:

After 24-hours of incubation, 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 LD_{50} values were determined using Probit analysis[12-13].

Selection of extraction solvent for bioactive compounds:

Bioactive compounds were isolated by applying different solvent system of different polarity. Solvent-solvent partitioning was done using the protocol designed by Kupchan and modified by Wagenen et al[14].

Extraction of compounds:

Extraction of bioactive compounds was performed by the method as described in the laboratory textbooks. 500 g of pumpkin seed was taken for extraction of bioactive compounds. The seeds were air dried at 20ºC and were crushed into fine powder by motor pastel .The powdered was poured into three large size reagent bottles. The powder was soaked by methanol (1 litter). Then the content of the bottle was shaken for seven days. After that the liquid portion was collected by filtering with cotton and finally by filter paper. The liquid portion was concentrated by rotary vacuum evaporator at 50ºC.The concentrated methanol extract was ready for further separation.[15-16].

Chromatographic analysis of crude petroleum ether extract:

Chromatographic analysis was performed to separate the compounds present in the crude extract and to isolate and purify them. For the purpose ascending one-dimensional thin layer chromatographic (TLC) technique was adopted using different solvent systems. Initially small plates were used to find out the most appropriate solvent system by which the compounds could be easily isolated and purified by TLC[17,18].

Saturation of TLC chamber:

Cylindrical glass chamber with airtight glass lid was used for the development of TLC plates. The selected mobile phase was poured into the chamber and a smooth sheet of filter paper was laid and soaked in the solvent. The chamber was then made air tight with the glass lid. It was kept for 20 to 30 minutes to saturate the internal atmosphere with the solvent vapor.

Resolution of the bioactive compounds:

The crude extracts were dissolved in respective solvent to make 1% (w/v) solution and were spotted with the help of a capillary tube on the activated TLC plates one centimeter apart from the bottom edge [19]. The spot was then dried with an air blower and a straight line was drawn two centimeters below the upper edge of the activated plate which marked the upper limit of the solvent flow. The spotted plate was then gently placed into the TLC chamber containing a selected solvent system in an inclined position so that the applied spot remained above the surface of the solvent. The chamber was closed with the lid and kept in an undisturbed position. When the solvent front reached the given mark, the plate was taken out and dried with a warm air blower.

Detection of bioactive compounds:

The following procedures were used to detect the bioactive compound in the TLC plates :

- Visual detection: The developed chromatogram was examined visually to detect the presence of colored bioactive compounds [20-21].
- Observation under UV light (254 nm**):** The chromatogram was examined under UV light to detect fluorescent compounds which exhibit glowing spots as well as pigment type compounds appearing as red spots. The distinct spots are marked [20-21].
- Iodine vapor: The developed chromatogram was exposed to iodine vapor in a closed jar containing few

crystals of iodine and kept for minutes. Air blower removed bound iodine from the plate [20-21].

- Vanillin sulfuric acid spry reagent: The chromatogram was sprayed with 1% vanillin -sulfuric acid reagent and then heated at 110°C for 10 minutes or until the spots attain maximum intensity. Steroid, terpene, glycosidic, heterocydic and flavonoid compounds produce pink, violet, black/gray, brown and yellow color respectively [20-21].
- Ehrlich's reagent: (0.5% para-dimethylaminobenzen-aldehyde) the plate was sprayed by this reagent and kept in HCl chamber for identification of limonoid[22].

Determination of R_f value (retardation factor) value of the resolved fraction:

R_f value is the characteristic of a compound in a specific solvent system. It helps in the identification of compounds. R_f value were calculated by the following formula:

R_f value =

$$\frac{\text{Distance travelled by the compound}}{\text{Distance travelled by the solvent (solvent front)}}$$

Isolation of the bioactive compounds in large amount by preparative thin layer chromatography (PTLC):

Preparation of PTLC:

A number of glass plates (20 cm × 20 cm) were used to have an absorbent layer of 0.5 mm thickness using slurry prepared by mixing silica gel GF_{254} (8 g/plate) with distilled water (2 ml/g of silica gel). The plates were air dried and then activated by heating at 110°C for 70 minutes.

Development of PTLC:

The petroleum ether extract (1% w/v) was applied on the plates as narrow bands with the help of a fine capillary tube (2 cm above the edge of the plate). About 105 ml of the solvent system n-hexane: ethyl acetate: methanol (9:1:.2) was poured into the cleaned and dried PTLC tank. Four filter papers were placed into tank and the tank was covered with glass lid. The tank was kept for 30 minutes to make its internal environment saturated with the solvent vapor. Four plates were then placed within the tank in the slanting position. When the solvent system reached the marked level, the plates were removed from the tank and air-dried.

Elution of compound from the identified bands:

The absorbed area was scrapped by means of a clean spatula and taken in a 100 ml beaker containing 44 ml of the established solvent system n-hexane: ethyl acetate: methanol (9:1:.2). This was stirred with a previously cleaned glass rod. After few minutes (15-20 min.) the content was passed through cotton plugged elution tube and solution was collected. The solvent was allowed to evaporate at room temperature and thus bioactive compounds were collected for further analysis.

Checking the purity of bioactive compounds:

The purity of isolated bioactive compound was checked by TLC using three different solvent systems. The isolated compounds were also chromatographed three times two dimensionally to check the purity of bioactive compounds.

An important tool of the organic chemist is Infrared Spectroscopy, or IR. IR spectra are acquired on a special instrument, called an IR spectrometer. IR is used both to gather information about the structure of a compound and as an analytical tool to assess the purity of a compound. Infrared refers to that part of the electromagnetic spectrum between the visible and microwave regions. Electromagnetic spectrum refers to the seemingly diverse collection of radiant energy, from cosmic rays to X-rays to visible light to microwaves, each of which can be considered as a wave or particle traveling at the speed of light .These waves differ from each other in the length and frequency.

The IR region is divided into three regions: the near, mid, and far IR. The mid IR region is of greatest practical use to the organic chemist. This is the region of wavelengths between 3 x 10–4and 3 x 10–3cm. Chemists prefer to work with numbers which are easy to write; therefore IR spectra are sometimes reported, although another unit,(nu bar or *wavenumber*), is currently preferred. Infrared radiation is absorbed by organic molecules and converted into energy of molecular vibration. In IR spectroscopy, an organic molecule is exposed to infrared radiation. When the radiant energy matches the energy of a specific molecular vibration, absorption occurs. A typical IR spectrum is shown below. The wavenumber, plotted on the X-axis, is proportional to energy; therefore, the highest energy vibrations are on the left. The percent transmittance (%T) is plotted on the Y-axis. An absorption of radiant energy is therefore represented by a "trough" in the curve: zero transmittance corresponds to 100% absorption of light at that wavelength. The wavenumbers (sometimes referred to as *frequencies*) at which an organic molecule absorbs radiation give information on functional groups present in the molecule. Certain groups of atoms absorb energy and therefore, give rise to bands at approximately the same frequencies. The chemist analyzes a spectrum with the help of tables which correlate frequencies with functional groups[23].

RESULTS AND DISCUSSION:

In brine shrimp lethality bioassay, the crude methanol extract (M) and petroleum ether fraction (PE) showed positive results indicating that the extractwere biologically active.

The LD_{50} values of M and PE of *cucurbita maxima* seeds were 31.70 and 21.95 ppm respectively, whereas positive control ampicillin trihydrate showed LD_{50} value of 21.37 ppm was shown in the table 1.1 and fig. 1.1-1.3. The mortality was not shown in the negative control experiment. From the above results it is evident that petroleum ether fraction (PE) is higher toxic than that of crude methanol extract and was nearer as ampicillin trihydrate. So we can conclude that the petroleum ether fraction (PE) of *Cucurbita maxima* seed have potent cytotoxicity.

The developed PTLC plates were visualized under UV light (254 nm). There were four fractions designated as PE-1, PE-2, PE-3 and. PE-4 and the R_f values of four fraction are 0.57, 0.75, 0.85 and 0.94, respectively. Only PE-4 band was isolated but other three bands, PE-1, PE-2 and PE-3 could not be isolated due to their long tail. PE-4 may be the steroid as it gave pink color by spraying with vanillin-sulfuric acid. The fraction gave a clear single spot on TLC. So it was subjected to IR spectroscopic analysis. The IR data showed that the isolated PE-4 compound contained aromatic ring and ketonic groups (-CO-). NMR and Mass spectral analysis is further needed to elucidate the molecular structure of the compound.

PE-4 may be steroid compound. IR data reveals that the compound contained an aromatic ring and ketonic Group (-CO-). Further investigation is needed to elucidate its structure.

CONCLUSION:

Cururbita maxima commonly known as Mistikumra, is cultivated all the districts of Bangladesh to satisfy nutritional requirements. Having database of the analysis of cultivated plants avialable in the region would be of value to educators and public health official positioned to provide dietary advice to the food stressed populations.

In Brine shrimp lethality bioassy indicate that petroleum fraction of seed has higher toxic effect than methanol extract. So, the petroleum ether fraction of seed might be used as larvicide, insecticidal, anticancer or antiviral agent. But it is needed for further investigation.

IR data reveals that the compound contained an aromatic ring and ketonic Group (-CO-). Further investigation is needed to elucidate its structure.

Table 1.1: Toxicity of crude methanol extracts and petroleum ether fraction (PE) of *Cucurbita maxima* against brine shrimp nauplii.

Sample	LD_{50} (ppm)	95% Confidence limit	Regression equation	Chi-squared χ^2
Ampicillin trihydrate	16.06	7.15-36.06	Y = 1.39596+ 3.3219 X	0.942190
Crude Methanol extract (M)	31.70	19.95689–50.36416	Y = 2.110196+1.925115 X	1.130755
Petroleum ether fraction (PE)	21.95	12.12193-39.76618	Y = 2.803507+1.637289 X	.3984318

Symbol: ppm = Chemical shift in parts per million.

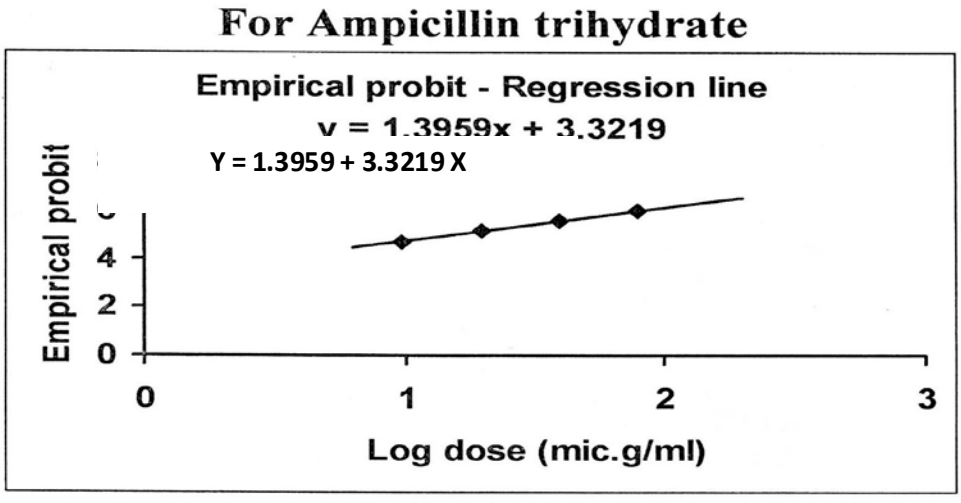

Figure 1.1: Regression line of log dose of ampicillin trihydrate against brine shrimp nauplii after 24h of exposure.

For Crude Methanol Extract

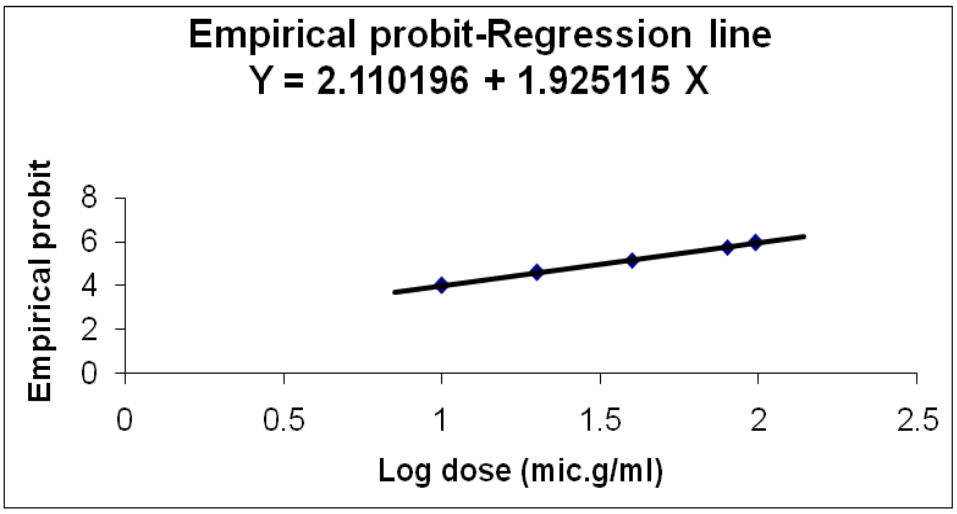

Figure 1.2: Regression line of log dose of crude methanol extract of *Cucurbita maxima* seed against brine shrimp nauplii after 24h of exposure.

For Petroleum Ether Fraction

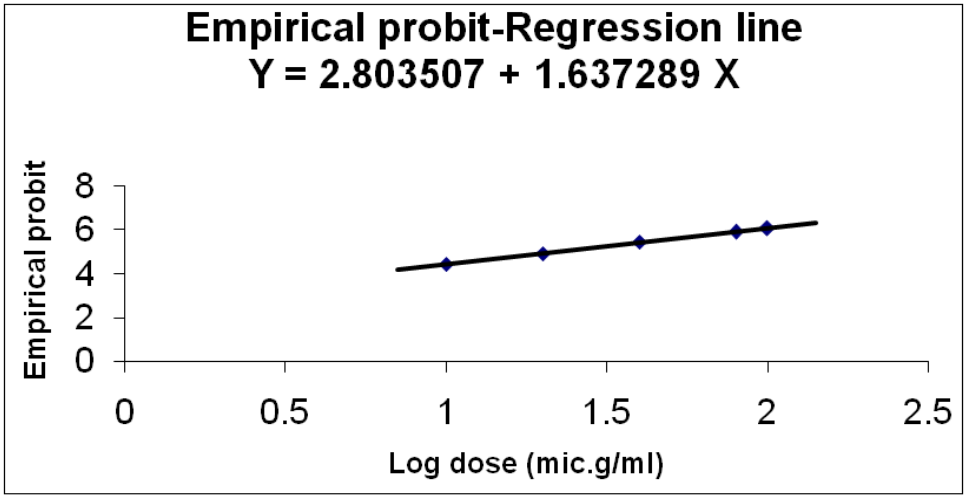

Figure 1.3: Regression line of log dose of petroleum ether fraction (PE) of *Cucurbita maxima* seed against brine shrimp nauplii after 24h of exposure.

Table 2.1: Components of petroleum ether fraction with R_f values resolved by TLC.

Component	Observation	R_f values
PE-1	Blackish in UV (254 nm) and binda with I_2 and black by spraying with vanillin sulfuric acid.	0.57
PE-2	Pink color by spraying with vanillin sulfuric acid.	0.75
PE-3	Reddish in UV (254 nm) and binds with I_2 and Pink color in (254 nm) and binds with I_2	0.85
PE-4	Pink color by spraying with vanillin sulfuric acid.	0.94

Figure 2.2: Resolution pattern of petroleum ether fraction on TLC plate.

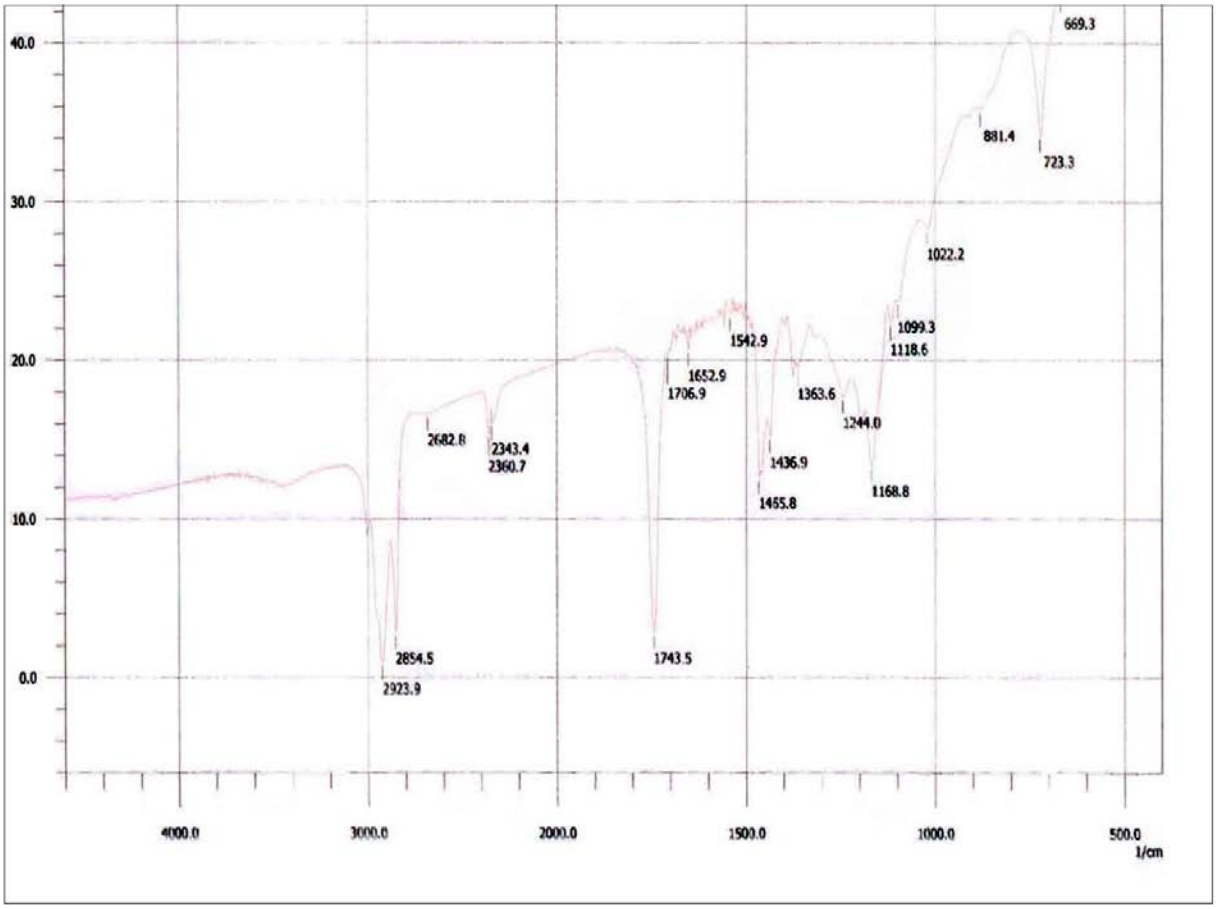

Figure 2.3: The IR data of PE-4 fraction.

Competing interest:
There is no conflict of interest

Acknowledgements:
This study was carried out in the Department of Biochemistry and Molecular Biology, Rajshahi University and was supported by the University Grant Commission (UGC), Bangladesh.

REFERENCE:

1. M. ObayedUllah, MahmudaHaque, Kaniz Fatima Urmi, AbuHasanat, Md. Zulfiker, Elichea SynthiAnita, Momtaj Begum, and Kaiser Hamid Anti-bacterial activity and brine shrimp lethality bioassay of methanolic extracts of fourteen different edible vegetables from Bangladesh.Asian Pac J Trop Biomed. Jan 2013; 3(1): 1–7.
2. Kaniecki, D. 2011.What Are the Medicinal Advantages of Pumpkin Seeds?(Accessed on Feb, 4, 2013).
3. Mclaughlin, J.L. and Anderson, J.E. Brine shrimp and crown gall tumors: simple bioassay for the discovery of plant antitumor agent. Proceedings NIH workshop.Bioassay for discovery of antitumor and antiviral agents from natural sources. Bethesda. pp22, (1988).
4. Mayer BN, Ferringni NR, Putnam JE, Jacobsen LB, Nichols DE, and Mclaughlin JL. Brine shrimp: a conveniant bioassay for active plant constituents. PlantaMedica. 45: 31-34 (1982).
5. Persoone G. *et al.*Proceeding the intrenational symposium on brine shrimp Artemia saline, volumes 1-3, Universe press. Witteren, Belgium 1-3, (1980).
6. Mclaughlin JL., Bench tops bioassay for the discovery of bioactive compounds in higher plants. Brenena.29, (1990).
7. Meyer BN, Ferrigni NR, Putnam JE, Jacobsen JB, Nicholsand DE, Mclaughlin JL. Brine shrimp; a convenient general bioassay for active plant constituents. Plant Med.1982; 45:31–34.
8. Sam TW. Toxicity testing using the brine shrimp: Artemiasalina. In: Colegate SM, Molyneux RJ, editors. Bioactive natural products detection, isolation, and structural determination. Boca Raton: CRC press; 1993. pp. 442–456.
9. Manilal A, Sujith S, Seghal KG, Selvin J, Shakir C. Cytotoxic potentials of red alga, laurenciabrandenii collected from the Indian coast. Global J Pharmacol.2009;3(2):90–94
10. Ali AM, Mackeen MM, Saleh H, Ei-Sharkawy, Hamid JA, Ismail NH, et al. Antiviral and cytotoxic activities of some plants used in Malaysian indigenous medicine. Pertanika J Trop Agric Sci. 1996;19(2/3): 129–136
11. Dissertation submitted in partial fulfillment of the requirements for the Degree of Master of Science in Life Science By Karmjit Singh Phytochemical determination and antibacterial activity of TrichosanthesdioicaRoxb (Patal),Cucurbita Maxima (pumpkin) and AbelmoschusesculentusMoench (Okra) plant seeds, Department of Life Science NATIONAL INSTITUTE OF TECHNOLOGY ROURKELA-769 008, ODISHA.
12. Finney, D. J., *Probit Analysis: a statistical treatment of sigmoid response curve.*Cambridge University Press, London, pp. 333, 1947.
13. Busvine, J.R., *A critical review of the techniques for testing insecticides:*Commonwealth Agricultural Buereux: London. pp. 345, 1971.
14. Wagenen, B.C. V, Larsen, R.J.H, Cardellina H. D, Randazza Z.C.L., Organic Chemistry, P. 58, 335, (1993).
15. Vogel, I.A., A Textbook of Quantitative Inorganic Analysis Including Elementary Instrumental Analysis, Longman London, 4th ed., p. 143 (1996).
16. Bahl, B.S. and Bahl, A., A Text Book of Organic Chemistry, S. Chand and Company Ltd., p. 5-6, 11-12, 14 (1992).
17. Stahl, Egon, Thin Layer Chromatography: A Laboratory Handbook "Revised and expanded", Siringer-Verlag, 2nd Ed, New York, (1969).
18. Remington's Pharmaceutical Sciences, Marc Publishing Company, 16th ed., p. 569-570 (1988).
19. Harborne, J.B, Encyclopedia, Methods: A Guide to Modern Technique of Plant Analysis, Reprin, Chapman and Hall Ltd., London, (1976).
20. Touchstone, J.C. and Dobbins, F.M. Practice of Thin Layer Chromatography, John Wiley and Sons Com. Ltd., 1sted, (1978).
21. Srivastava, V.K. and Srivastava, K.K, An Introduction to Chromatography, Theory and Practice, p. 5, 50-52, (1987).
22. Mercr E, Dyeing Reagents for Thin Layer Chromatography, p. 39, (1980).
23. Online edition for students of organic chemistry lab courses at the University of Colorado, Boulder, Dept of Chem and Biochem. (2002)

