

Original Article

IN VITRO ANTIOXIDANT AND ANTICANCER ACTIVITY OF *MIMOSA PUDICA* LINN EXTRACT AND L-MIMOSINE ON LYMPHOMA DAUDI CELLS

FELISA PARMAR, NISHA KUSHAWAHA, HYACINTH HIGHLAND, LINZ-BUOY GEORGE

Department of Zoology, Biomedical Technology and Human Genetics, University School of Sciences, Gujarat University, Ahmedabad 380009, Gujarat, India
Email: felisaparmar@yahoo.com

Received: 06 Aug 2015 Revised and Accepted: 27 Oct 2015

ABSTRACT

Objective: The present study is an attempt to investigate the antioxidant and anticancer potential of hydroalcoholic extract of *Mimosa pudica* Linn (Mimosaceae) and L-Mimosine on Daudi cell line.

Methods: The analysis of the standard compound L-Mimosine was ascertained by High Performance Thin Layer Chromatography (HPTLC). Free radical scavenging activity of *M. pudica* extract and L-Mimosine was also compared using 2, 2-diphenyl-1-picrylhydrazyl radical scavenging assay (DPPH). Cell viability and cytotoxicity on Daudi cells were evaluated by trypan blue and 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assays in a dose and time-dependent manner respectively.

Results: HPTLC analysis showed the presence of amino acids, amines, lipids in the hydroalcoholic extract of *M. pudica*. Crude hydroalcoholic extract of *M. pudica* showed antioxidant activity ($IC_{50}=103.88 \mu\text{g/ml}$) whereas L-Mimosine showed antioxidant activity ($IC_{50}=233.06 \mu\text{M}$). The crude extract and compound inhibited the proliferation and growth of the Daudi cells through induced cell death. The IC_{50} value for anticancer activity was found to be 201.65 $\mu\text{g/ml}$ and 86.61 μM at 72 h for *M. pudica* extract and L-Mimosine respectively.

Conclusion: The results indicated the presence of L-Mimosine in the hydroalcoholic extract of *M. pudica*. The hydroalcoholic extract and pure compound proved potent inhibitors of cell proliferation, thus manifesting significant antioxidant and anticancer activity.

Keywords: Anticancer, Antioxidant, Daudi, *Mimosa pudica*, L-Mimosine, HPTLC.

INTRODUCTION

Cancer has been one of the most dreaded diseases of the 20th century and is increasing rampantly with greater intensity in the 21st century. Cancer in adolescent and young adults is 2.7 times more common than cancer occurring during the first 15 y of life. In females aged between 15 to 29 y, malignancies of the genital tract are the most frequent type of cancer (18%), followed by lymphoma (17%) [1].

Over the last few decades, there has been increased interest by pharmaceutical industries to discover new drugs from the ethnobotanicals to provide new and alternative drugs to synthetic drugs for the treatment of dreadful diseases. Only a few plants have been significantly explored regarding their medicinal uses.

Antitumor drug resistance and side effects of antitumor compounds are the most common hurdles in medicine [2]. Plants are rich sources of anticancer agents and their derivatives are very useful for the treatment or prevention of cancer. These plant compounds such as vinblastine have been demonstrated as therapeutic for Hodgkin's lymphoma and vincristine for non-Hodgkin's lymphoma while Camptotheca acuminata, topotecan and irinotecan have been used for lung and ovarian cancer [3]. Anticancer activity is the effect of natural and synthetic or biological and chemical agents to reverse, suppress or prevent carcinogenic progression. Several synthetic agents are used to cure the disease but they have their cytotoxicity and hence the research is now focused towards investigating plant derived chemotherapeutic agents.

Mimosa pudica known as "Chue Mue" belongs to taxonomic group Magnoliopsida and family Mimosaceae, is a stout straggling prostrate shrubby plant, with compound leaves that get sensitive on touching, pinous stipules and globose pinkish flower heads; it grows as a weed in almost all parts of the country. Leaves and stems of the plant have been reported to contain an amino acid Mimosine. The leaves also contain mucilage and roots contain tannins [4]. *M. pudica* leaves are used for its antihyperglycaemic, antiulcer, anti diarrheal, anticonvulsant, cytotoxic and hepatoprotective properties. Moreover,

Paranjpe (1989) has reported that in Ayurvedic and Unani medicine, *M. pudica* root is used to treat bilious fever, piles, jaundice, leprosy, dysentery, vaginal and uterine complaints, inflammations and burning sensation, fatigue, asthma, leucoderma and blood diseases [5].

L-Mimosine or leucenol [$C_8H_{10}N_2O_4$, β -N-(3-hydroxy-4-pyridone)-amino propionic acid] is an amino acid present in plants of genus *Mimosa* and *Leucaena* [6]. This rare amino acid exhibits a wide range of effects, including inhibition of folate metabolism [7], inhibition of deoxyribonucleotide metabolism [8], induction of apoptosis [9, 10], inhibition of different cell line proliferation and *in vivo* inhibition of tumor growth [11, 12]. It has shown an inhibitory effect on different mammalian enzymes such as tyrosinase, dopamine β -hydroxylase and deoxyhypusine hydroxylase [13, 14]. The aim of the current study is therefore to identify natural compounds that can be used for prevention or treatment of cancer.

The cell line used in this study was Daudi cells which were cultured from a line of lymphoblastoid cells derived from a human Burkitt (non-Hodgkin) lymphoma which is cancer of the lymphatic system. We have studied the effect of hydroalcoholic extract of *M. pudica* and L-Mimosine on Daudi cells *in vitro*.

MATERIALS AND METHODS

Chemicals

L-Mimosine was purchased from MP Biomedicals (France) whereas all other chemicals were purchased from Hi-Media Laboratories (India).

Preparation of plant extracts

The plant of *M. pudica* was collected from Gujarat University campus and authenticated by Dr. H. A. Solanki, Professor, Botany Department, Gujarat University, Ahmedabad. The whole plant viz., stems, leaves, roots and flower buds of *M. pudica* were collected, washed and shade dried under ambient temperature. After complete drying plants were powdered and defatted with petroleum ether (40-60 °C) and kept for 24 h at room temperature with constant shaking. 50 gm of the defatted powdered material was capsulated in

filter paper and kept in the thimble, 500 ml solvent (water: ethanol) (70:30) was added into the flask and continuous extraction was carried out in the Soxhlet apparatus for 72-74 h at 60 °C, (till the colour in the siphon became colourless). The crude solvent collected in the flask was dried at reduced pressure and kept at 4 °C until further use.

DPPH radical scavenging activity

The antioxidant activity of plant extract and L-Mimosine was measured *in vitro* using DPPH, a stable free radical. The reaction mixture contained 0.1 ml of 0.1 mM DPPH and 0.1 ml of *M. pudica* extract (31.25-250 µg/ml) or L-Mimosine (31.25-250 µM). The solution was mixed rapidly and allowed to stand for 30 min in the dark. The scavenging activity was measured by noting the decrease in absorbance at 520 nm as compared to DPPH control. The analysis was done in triplicate. The assay procedure was followed from those described by Blois (1958) and Yamasaki *et al.*, (1994) [15,16]. Inhibition of free radical DPPH in percent (I %) (or) the DPPH free radical scavenging activity (%) was calculated from the absorption according to the following equation:

$$I (\%) \text{ (Or) DPPH Scavenged } (\%) = \frac{\text{Absorbance of control} - \text{Absorbance of sample}}{\text{Absorbance of control}} \times 100$$

Dose response curve was plotted between % inhibition and concentrations. IC₅₀ values were found out for plant extract as well as for compound.

HPTLC

The extract was first run along with the standard compound on Thin Layer Chromatography (TLC) plate through Phenol-Ethanol-Water (3:1:1) and spots were developed by ninhydrin spraying reagent (3% in acetone) [17]. This was then followed by scanning, determination of retention factor (*R_f*) value and λ Max profile [18] by Camag-5 HPTLC system WinCATS evaluation software (version 1.4.6.8121).

Cancer cell culture

For cancer cell culture, Daudi cell line was obtained from the National Centre for Cell Science (NCCS), Pune. Cells were cultured in RPMI 1640 media with 10% fetal bovine serum (FBS) and antibiotic-antimycotic solution. Cell cultures were maintained in a CO₂ incubator at 5% CO₂ and 37 °C.

Cell viability assays by trypan blue dye exclusion technique

Any compound, which is cytotoxic to cells, inhibits the cell growth proliferation and kills the cells. Trypan blue is a supravital dye, used to estimate the number of cells present in the population [19]. It has the ability to penetrate dead cells and give it a blue colour. This method gives a score of dead and viable cells [20].

Cellular cytotoxicity induced by the *M. pudica* extract and L-Mimosine was measured with trypan blue exclusion assay. Sterility was maintained throughout the procedure. Briefly, 2×10⁶ cells were seeded into 24-well plates and treated with or without (as control) crude hydroalcoholic extract of *M. pudica* (12.5-400 µg/ml) and L-Mimosine (12.5-400 µM) for 24, 48 and 72 h. After the incubation period, the cultures were harvested and washed twice with Phosphate Buffered Saline (PBS). The cell pellet was then resuspended with 0.5 ml PBS. Then, 20 µL of cells were mixed with equal volume of 0.4% trypan blue and was counted using a Neubauer hemocytometer by clear field microscopy. Viable and non-viable cells were counted. The percentage cytotoxicity was calculated using the equation shown below:

$$\% \text{ Viability} = \frac{\text{Live cell count}}{\text{Total cell count}} \times 100$$

In vitro cytotoxicity determination by MTT assay

The ability of cells to survive a toxic insult is the basis of most cytotoxic assays. This assay is based on the assumption that dead

cells or their products do not reduce tetrazolium. It is described by the modified method of Mosmann, (1983) and Wilson *et al.*, (2000) [21, 22].

The assay detects the reduction of MTT by mitochondrial dehydrogenase to blue formazan product, which reflects the normal function of mitochondria. 2×10⁶ viable cells/ml were plated into the 96-well cell culture plate. The crude extract was added with the concentrations (12.5-400 µg/ml) and compound with (12.5-400 µM) respectively for 24, 48 and 72 h and incubated at 37 °C. After incubation, the supernatants were removed and incubated with MTT (0.5 v/v) in RPMI 1640 without FBS for 4 h in a humidified atmosphere at 37 °C and 5% CO₂ incubator.

The absorbance (A) of the coloured solution was quantified at 540 nm wavelengths by an enzyme-linked immunoabsorbent assay reader (ELISA READER, MERCK MIOS mini). Each extract, compound and control was assayed in triplicate in three independent experiments. Percent growth inhibition of cells exposed to treatments was calculated as follows:

$$\% \text{ Inhibition} = \left(100 - \left(\frac{\text{Corrected mean Absorbance of sample}}{\text{Corrected mean Absorbance of control}} \right) \right) \times 100$$

Statistical analysis

Each parameter was performed in triplicate and the results were expressed as mean±standard error. The data was statistically analyzed by Student's 't' test and the values of p<0.05 were considered statistically significant.

RESULTS

Antioxidant effect *M. pudica* and L-Mimosine

The DPPH radical scavenging activity of extract and L-Mimosine is shown in the fig. 1. It was observed that DPPH free radical scavenging activity was concentration dependent in both the cases and reaches a maximum at a concentration of 250 µg/ml for plant extract and 250 µM for the compound. Our result indicated that *M. pudica* extract exhibited high antioxidative activity compared to L-Mimosine.

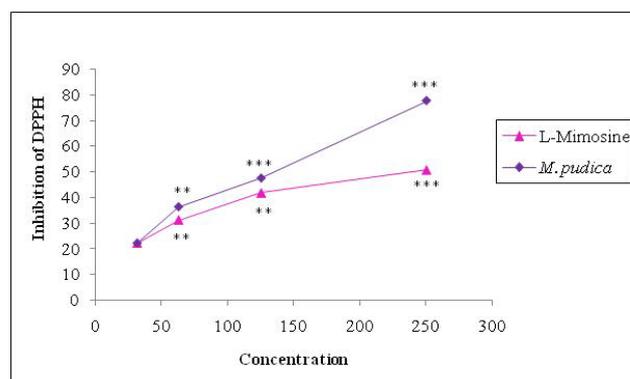


Fig. 1: DPPH scavenging activity of *Mimosa pudica* with IC₅₀ value of 103.88 µg/ml and L-Mimosine with IC₅₀ value of 233.06 µM

Values are mean±S.E. for three individual experiments.

HPTLC

The peak heights area of the respective L-Mimosine standard and the hydroalcoholic plant extract are shown in fig. 2 and 3. The hydroalcoholic plant extract showed ten prominent spots on the TLC plate with a retention factor of 0.09, 0.12, 0.21, 0.29, 0.45, 0.52, 0.62, 0.67, 0.84 and 0.94 (fig.3). HPTLC determination showed the presence of amino acids, amines, lipids in the hydroalcoholic extract of *M. pudica* and it was confirmed from the chromatogram after derivatization with Ninhydrin spraying reagent (fig. 4).

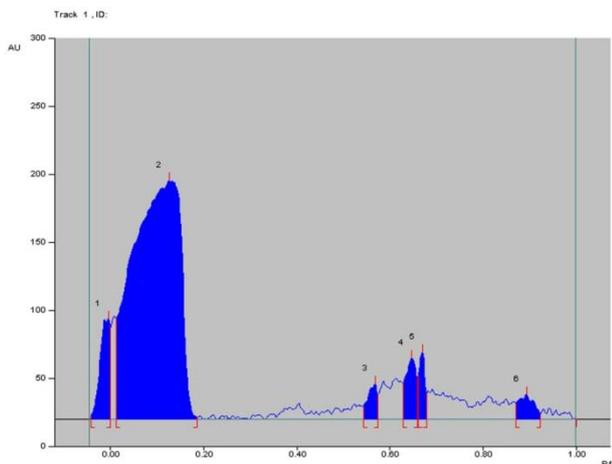


Fig. 2: HPTLC chromatogram area and peaks of the L-Mimosine using Win CATS evaluation software (version 1.4.6.8121)

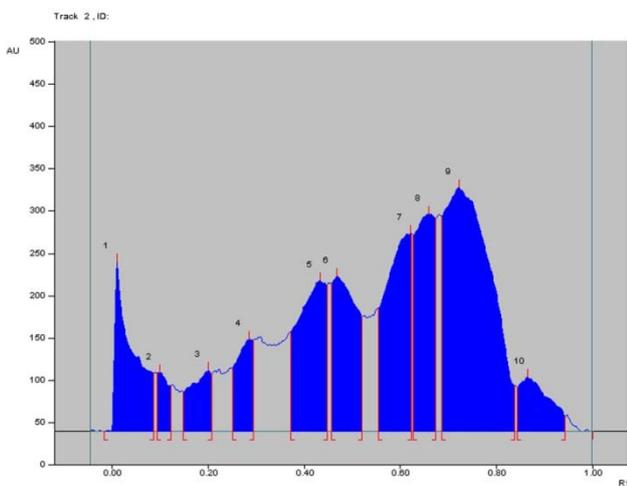


Fig. 3: HPTLC chromatogram area and peaks of the *Mimosa pudica* extract using Win CATS evaluation software (version 1.4.6.8121)

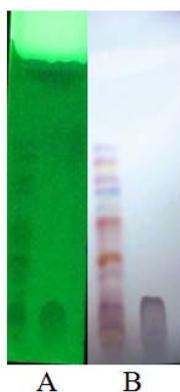


Fig. 4: Thin layer chromatogram of *Mimosa pudica* and L-Mimosine using the solvent system Phenol: Ethanol: Water (3:1:1 v/v/v) under 254 nm (A) and visible light (B)

Cell viability assay

The cell viability assay conducted by trypan blue dye exclusion method showed that there was a highly significant ($p < 0.001$) decrease in viability with an increase in time and concentration in

both the extract as well as pure compound treated Daudi cells as compared to untreated controlled cells.(fig. 5 and 6).

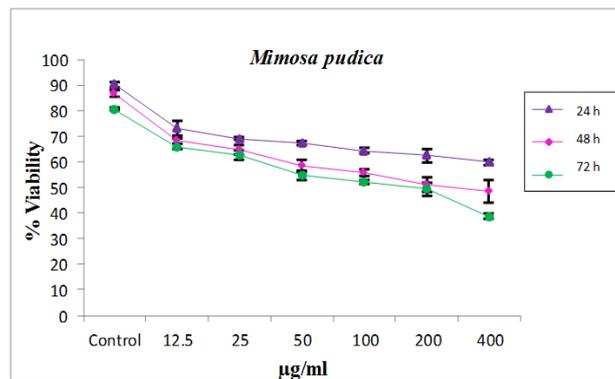


Fig. 5: Effect of *Mimosa pudica* extract on percentage viability on Daudi cells. Values are mean±SE for three individual experiments

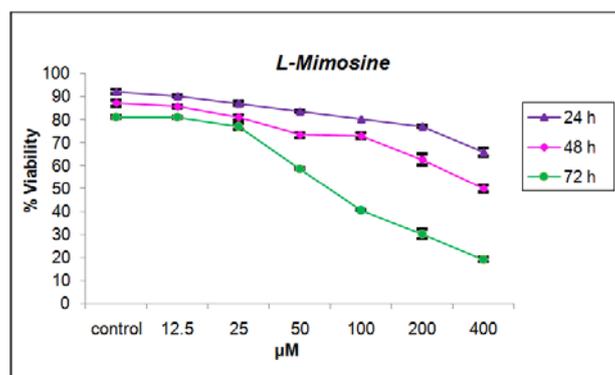


Fig. 6: Effect of L-Mimosine on percentage viability on daudi cells. Values are mean±SE for three individual experiments. Except for treatment with 12.5 µM and 25 µM, there is a highly significant ($p < 0.001$) decrease of cell viability in all treated cells

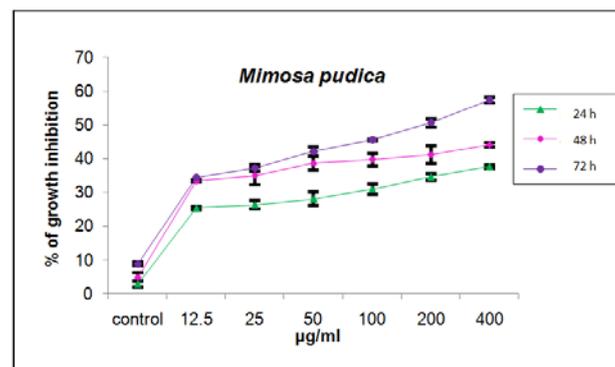


Fig. 7: Effect of *Mimosa pudica* extract on the percentage decrease of growth proliferation in Daudi cells. Values are mean±SE for three individual experiments. Highly significant ($p < 0.001$) increase in growth inhibition was observed in all the treated samples

MTT assay

Daudi cells were grown in 96 well plates for 24, 48 and 72 h with different concentration (12.5-400 µg/ml) of the crude plant extract and (12.5-400 µM) L-Mimosine compound. The formazan crystals were formed, following the reduction of MTT by metabolically active

(viable) cells. The percentage decrease of proliferation after treatment with the *M. pudica* and L-Mimosine is given in Fig.7 and 8 respectively compared to control. The IC₅₀ value was calculated by plotting the graph in Microsoft Excel and was found to be 201.65 µg/ml and 86.61 µM at 72 h for *M. pudica* extract and L-Mimosine respectively. There was a significant increase in the percentage of inhibition of growth proliferation with increased dose and time duration as compared to untreated control cells ($p < 0.001$).

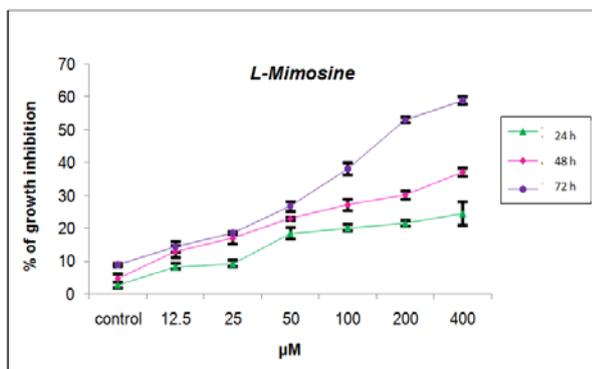


Fig. 8: Effect of L-Mimosine extract on the percentage decrease of growth proliferation in Daudi cells. Values are mean±SE for three individual experiments. Highly significant ($p < 0.001$) increase in growth inhibition was observed in all the treated samples

DISCUSSION

The greatest challenge for phytochemical and pharmacological studies involves the identification of the specific compounds that are responsible for the beneficial effects and their modes of action, thereby delineating their useful functions as therapeutic drugs. *Mimosa pudica* Linn is a well-known herbal medicine throughout the world. Many studies have reported the pharmacological efficacies and benefits of *M. pudica*. Jose *et al.* (2014) have shown potential anticancer activity with isolated flavonoids of *M. pudica* against MCF-7, human breast cancer cell line. Cytotoxic activity of *M. pudica* was evaluated using brine shrimp lethality bioassay which had shown little cytotoxic effect and results suggest that the plant can be used as a promising source of anticancer compounds [24]. There is very little information available on its mechanism of action on cancer cells. On the other hand, Lalande and Hanauske-Abel (1990) have shown that Mimosine induces cell-arrest (reversibly) late in the G1 phase of the cell cycle.

Moreover, Mimosine is known to be a tyrosine analog that contains a metal chelating domain and hence it is able to chelate transition metals, such as Fe³⁺ (Linn *et al.* 1996). The possible structure of the Fe (III)-Mimosine chelation complex has also been proposed by Tsai and Ling (1973). Furthermore, there is evidence that Mimosine inhibits various mammalian enzymes *in vitro*, such as tyrosinase, dopamine hydroxylase [13], deoxyhypusyl hydroxylase (DOHH) [28], and H1 kinase [29]. These results strongly suggest that Mimosine may inhibit the intense mitotic activity of cancer cells. In fact, *in vitro* studies have demonstrated that Mimosine represses uterine cancer cell growth [30] and blocks DNA replication in both breast cancer and Chinese hamster ovary cells [31]. It has been shown that Mimosine acts as an iron chelator [32] and the resulting iron deficiency could alter folate metabolism in mammals and interfere with tumor cell growth [33]. The DPPH assay is based on the reduction of stable radical DPPH to yellow coloured diphenyl picryl hydrazine. Thus, the ability of the tested products to quench this radical is a measure of its antioxidative ability. Many previous studies have reported strong to the moderate free radical activity of crude extracts of the plants belonging to Mimosaceae. Normal cells have been studied previously in the same experiment and no significant effect has been found on normal cells.

DPPH has been used to evaluate the free radical scavenging activity of the natural antioxidant. DPPH which is a radical itself with a yellow color, changes into a stable compound antioxidant and the

extent of the reaction depends on the hydrogen donating ability of the antioxidant [34]. Both extract and compound showed potent free radical scavenging activity with IC₅₀ value of 103.88 µg/ml and 233.06 µM respectively. The ability of the extract of *M. pudica* and L-Mimosine to scavenge DPPH radicals suggests that it can react with free radicals to convert them to more stable products and terminate radical chain reaction.

In this study, hydroalcoholic extract of *M. pudica* and L-Mimosine were used to evaluate their possible anticancer activity. Both *M. pudica* hydroalcoholic extract and L-Mimosine treatment caused a significant loss of viability of cells as measured by this assay in a dose and time-dependent manner respectively.

The cytotoxic activity was monitored by the standard MTT assay. The ability of cells to survive a toxic insult is the basis of most repeated cytotoxic assays. This assay depends on both on the mitochondrial activity per cell and number of cells present. The cleavage of MTT to a blue formazan derivative by living cells is clearly a very effective principle on which the assay is based conforming the decrease in survival of the cultured cells.

The loss of viability and anticancer effect of L-Mimosine on Daudi cell line is might be due to inhibition of cellular enzymes *in vitro* [30].

CONCLUSION

The present study reported L-Mimosine from *Mimosa pudica*. Present data indicate that L-Mimosine treatment exhibited less antioxidant activity. Treatment with both hydroalcoholic extracts of *M. pudica*/ L-Mimosine showed potent anticarcinogenic effects against Daudi Lymphoma cell line leading to anti proliferation and loss of cell viability. To conclude data suggested that L-Mimosine treatment exhibited much higher anticarcinogenic activity compared to the extract of *M. pudica*.

CONFLICT OF INTERESTS

The authors have declared no conflict of interests.

REFERENCES

- Bleyer A, Viny A, Barr R. Cancer in 15-to 29-year-olds by primary site. *Oncologist* 2006;11:590-601.
- Holohan C, Schaeysbroeck SV, Long ley DB, Johnston PG. Cancer drug resistance: an evolving paradigm. *Nat Rev Cancer* 2013;13:714-26.
- Cragg GM, Newman DJ. "Plants as a source of anticancer agents,". *J Ethnopharmacol* 2005;100:72-9.
- Ghani A. Medicinal plants of Bangladesh with chemical constituents and uses. ed 2. Dhaka, Bangladesh; 2003. p. 302-3.
- Paranjpe P. Indian medicinal Plants: Forgotten healers. Delhi, India; 1989. p. 155-6.
- Thompson JF, Morris CJ, Smith IK. New naturally occurring amino acids. *Annu Rev Biochem* 1969;38:137-58.
- Oppenheim EW, Nasrallah IM, Matri MG, Stover PJ. Mimosine is a cell-specific antagonist of folate metabolism. *J Biol Chem* 2000;275:19268-74.
- Gilbert DM, Neilson A, Miyazawa H, De Pamphilis ML, Burhans WC. Mimosine arrests DNA synthesis at replication forks by inhibiting deoxyribonucleotide metabolism. *J Biol Chem* 1995;270:9597-606.
- Reno F, Tontini A, Burattini S, Papa S, Falcieri E, Tarzia G. Mimosine induces apoptosis in the HL60 human tumor cell line. *Apoptosis* 1999;4:469-77.
- Zalatnai A, Bocsi J. Mimosine, a plant-derived amino acid induces apoptosis in human pancreatic cancer xenografts. *Anticancer Res* 2003;23:4007-9.
- Chang HC, Weng CF, Yen MH, Chuang LY, Hung WC. Modulation of cell cycle regulatory protein expression and suppression of tumor growth by mimosine in nude mice. *Int J Oncol* 2000;17:659-65.
- Ju H, Hao J, Zhao S, Dixon IM. Antiproliferative and antifibrotic effects of mimosine on adult cardiac fibroblasts. *Biochim Biophys Acta* 1998;1448:51-60.
- Hashiguchi H, Takahashi H. Inhibition of two copper-containing enzymes tyrosinase and dopamine beta hydroxylase, by L-mimosine. *Mol Pharmacol* 1977;13:362-7.

14. Hanauske-Abel HM, Park MH, Hanauske AR, Popowicz AM, Lalande M, Folk JE. Inhibition of the G1-S transition of the cell cycle by inhibitors of deoxyhypusine hydroxylation. *Biochim Biophys Acta* 1994;1221:115-24.
15. Blois MS, Zhao XY. Antioxidant determinations by the use of stable free radicals. *Nature* 1985;181:1199-200.
16. Yamasaki K, Hashimoto A, Kokusenya Y, Miyamoto T, Sato T. Electrochemical method for estimating the antioxidative effects of methanol extracts of crude drugs. *Chem Pharm Bull* 1994;42:1663-5.
17. Spenser ID, Notation AD. A synthesis of mimosine. *Can J Chem* 1962;40:1374-79.
18. Sharma A, Patel VK. *In vitro* screening of the antibacterial activity and identification of bioactive compounds from plants against selected *Vibrio* spp. pathogens. *Turk J Biol* 2009;33:137-44.
19. Phillips HJ, Terryberry JE. Counting actively metabolizing tissue cultured cells. *Exp Cell Res* 1957;13:341-7.
20. Kuttan R, Bhanumathly P, Nirmala K, George MC. Potential anticancer activity of turmeric (*Curcuma longa*). *Cancer Lett* 1985;29:197-202.
21. Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and Cytotoxicity assays. *J Immunol Methods* 1983;65:55-63.
22. Wilson AP. Cytotoxicity and viability Assay in Animal Cell culture, A Practical Approach. ed 3. New York, NY; 2000. p. 175-219.
23. Jose J, Sudheesh S, Sumesh Kumar TM, Sony J, Jayadevi Variyar E. A comparative evaluation of anticancer activities of flavonoids isolated from *Mimosa pudica*, *Aloe vera*, *Phyllanthus niruri* against human breast carcinoma cell line (MCF-7) using MTT assay. *Int J Pharm Pharm Sci* 2014;6:319-22.
24. Srikanta C, Dibyajyoti S, Swati P. *In vitro* cytotoxic activities of methanolic extracts of *Mimosa pudica*. *Bull Pharm Res* 2012;2:42-5.
25. Lalande M, Hanauske-Abel HM. A new compound which reversibly arrests T lymphocyte cell cycle near the G1/S boundary. *Exp Cell Res* 1990;188:117-21.
26. Linn HB, Falchetto R, Mosca PJ, Shabanowitz J, Hunt DF, Hamlin JL. Mimosine targets serine hydroxymethyltransferase. *Biol Chem* 1996;271:2548-56.
27. Tsai WC, Ling KH. Study on the stability constant of some metal ion chelate of mimosine and 3,4-tihydroxypyridine. *J Chin Biochem Soc* 1973;2:70-86.
28. Abbruzzese A, Hanauske AHM, Park MH, Henke S, Folk JE. The active site of deoxyhypusyl hydroxylase: use of catechol peptides and their component chelator and peptide moieties as molecular probes. *Biochim Biophys Acta* 1991;1077:159-66.
29. Feldman ST, Schonthal A. Negative regulation of histone H1 kinase expression by mimosine, a plant amino acid. *Cancer Res* 1994;54:494-8.
30. Zalatnai A. P-glycoprotein expression is induced in human pancreatic xenografts during treatment with a cell cycle regulator, mimosine. *Pathol Oncol Res* 2005;11:164-9.
31. Mosca PJ, Dijkwel PA, Hamlin JL. The plant amino acid mimosine may inhibit initiation at origins of replication in Chinese hamster cells. *Mol Cell Biol* 1992;12:4375-83.
32. Kulp KS, Vulliet PR. Mimosine blocks cell cycle progression by chelating iron in asynchronous human breast cancer cells. *Toxicol Appl Pharmacol* 1996;139:356-64.
33. Gilbert DM, Neilson A, Miyazawa H, Depamphilis ML, Gupta HK, Altreja PP. Influence of ferric chloride treated *Leucaena leucocephala* on metabolism of mimosine and 3-hydroxy-4 (1H)-pyridone in growing rabbits. *Anim Feed Sci Technol* 1998;74:45-55.
34. Chen CW, Ho CT. Antioxidant properties of polyphenols extracted from green and black teas. *J Food Lipids* 1995;2:35-46.