Antiproliferative activity and phytochemical analysis of methanol leaf extract of *Grewia nervosa*

Grewia nervosa belonging to the Malvaceae family is abundantly found in the Western Ghats of India. Though *G. nervosa* is considered to have therapeutic potential, this has not been scientifically corroborated. However, the plant has been scientifically proven to demonstrate insecticidal, larvicidal and free radical scavenging activities[1–3].

Cancer is a pandemic disease and a major cause of death all over the world. Around 100,000 new patients are annually diagnosed with breast cancer in India[4]. In contrast to side effects allied with synthetic chemotherapeutic agents, the therapeutic effects provided by herbal medicine devoid of any side effects have augmented their exploitation in cancer treatment. With the advent of several *in vitro* tools, several plants are being regularly screened for the treatment of various diseases. The calorimetric-based automated screening bioassay is one such tool that is widely employed for evaluating drugs that inhibit cell proliferation[5].

The traditional ethno-pharmacological significance of medicinal plants acknowledged as folk remedies could be further evaluated by identifying the active ingredients and associating them with the medicinal properties. Gas chromatography-mass spectrometry (GC-MS) is routinely used for qualitative and quantitative analysis of plant secondary metabolites such as alkaloids, flavonoids, sterols, phenolics, alcohols, volatile oils, nitrogen containing compounds, etc.[6].

The present study evaluates the antiproliferative and cytotoxic effect of methanol leaf extract (MLE) of *G. nervosa* on MCF-7 breast cancer cell line. To assess additional oblivious pharmacological properties of *G. nervosa*, the volatile fractions in MLE were analysed by GC-MS to identify the prospective compounds that can be associated with anticancer and other medicinal properties.

Leaves of *G. nervosa* used in the present study were obtained from the campus of Goa University (15°27′35.05″N, 73°49′58.46″E, 52 m). The taxonomic identity of the plant was confirmed by M. K. Janarthanam (Department of Botany, Goa University) and the voucher specimen (sample collection no. 5312) was deposited in the herbarium. The air-dried leaves of *G. nervosa* (9 g) were extracted with methanol or distilled water using soxhlet extractor for 24 h at 65°C. The MLE or aqueous leaf extract (ALE) was further concentrated using a rotary evaporator to produce dry powder that was suspended in methanol or distilled water at the desired concentration and used immediately for bioassay.

The MTT assay was used to study the effect of MLE or ALE on proliferation of MCF-7 cell line. Cells (1×10^3) were seeded in a 96-well plate (triplicates) containing RPMI-1640 medium supplemented with 10% foetal bovine serum. Before addition of plant extracts (0 day), the plates were incubated at 37°C in a humidified incubator that was saturated with 5% CO_2. After 24 h, 200 or 400 μg ml^{-1} of MLE or 200 μg ml^{-1} of ALE was added and incubated for 5 days. Cell proliferation and cytotoxicity of plant extracts on MCF-7 cells were determined on day 0, 3 and 5. After addition of MTT dye to the wells, the plates were further incubated for 1 h at 37°C. MTT solution from each well was replaced by the addition of DMSO. Cell proliferation was determined at 595 nm using a plate reader. The % cell viability of MCF-7 cell line was obtained using the formula

Viability % = (Optical density of sample/optical density of control) × 100.

Alternatively, cell viability of MCF-7 cell line was also determined at various concentrations of MLE. Cells grown in RPMI-1640 medium without MLE or ALE were taken as control. After correlating the optical density of the test sample with control, the calculated cytotoxicity values were used to estimate the concentration of MLE that resulted in 50% reduction (IC_{50}) in growth (cell number) of cell line.

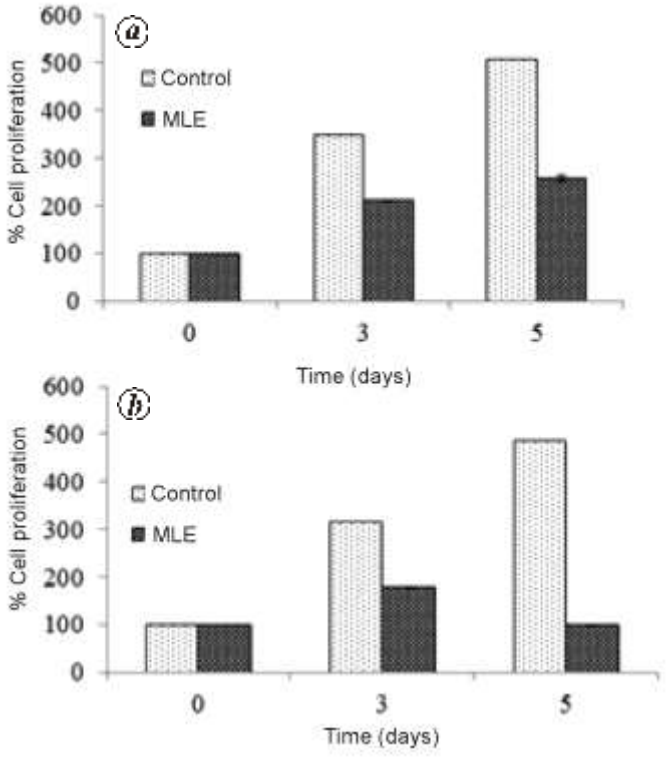

Figure 1. Effect of methanol leaf extract of *Grewia nervosa*: (***a***) 200 μg ml^{-1} and (***b***) 400 μg ml^{-1}, on proliferation of MCF-7 breast cancer cell line.

For trypan blue assay, MCF-7 cells (1×10^3 cells per well in a 96-well plate) were seeded in triplicate. Initially the cells were grown in RPMI-1640 medium supplemented with 10% foetal bovine serum at 37°C in an incubator that was saturated with 5% CO_2 in the absence of MLE. After 24 h, 200 or 400 µg ml^{-1} of MLE was added to the MCF-7 cell lines and viability of the cells was estimated by trypan blue on the third and fifth day respectively. For trypan blue assay, a 1 : 1 dilution of cell suspension was made in a 0.5% trypan blue solution. Cell counting was done using hemocytometer under compound microscope. Cell death was estimated by the relation

Cell death % = (Optical density of sample/optical density of control) × 100.

All the experiments were performed in triplicate and standard deviation was calculated for each data and represented as mean ± standard deviation.

GC-MS study was accomplished using GC SHIMADZU QP 2010 system, Japan. The temperature maintained at ion source and injector was 200°C and 250°C respectively. The temperature of the oven was programmed from 40°C (isothermal for 3 min), with an increase to 280°C for 10 min. Total GC running time was 40 min. Mass spectra were taken at 0.60 kV at 0.5 sec interval with scan in the range 35–500 m/z. The spectra were compared with NIST mass spectral library (2013) as well as the Wiley Registry for identification of the phytochemicals.

Table 1. Major phytochemicals detected in the methanol leaf extract of *Grewia nervosa* by GC-MS (peak areas >4% are shown)

Retention time (min)	Peak area (%)	IUPAC name	Molecular weight (g/mol)
16.57	4.61	*n*-Hexadecanoic acid	256
17.99	14.25	Phytol	296
18.13	8.20	α-Linolenic acid	278
18.28	16.39	9-Isopropyl-1-methyl-2-methylene-5-oxatricyclo[5.4.0.0(3,8)] undecane	220
26.60	20.48	α-Tocopherol	592
29.28	4.48	γ-Sitosterol	414

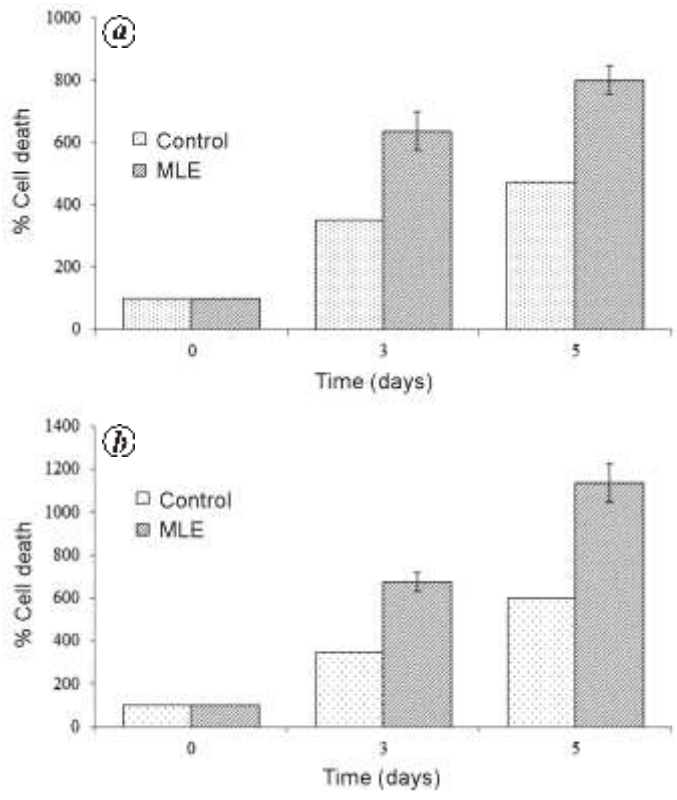

Figure 2. Effect of methanol leaf extract of *G. nervosa*: (***a***) 200 µg ml^{-1} and (***b***) 400 µg ml^{-1}, on cell viability of MCF-7 breast cancer cell line.

MLE of *G. nervosa* demonstrated concentration as well as time-dependent suppression of MCF-7 cell proliferation. In comparison to control (without plant extract), cell line incubated with 200 µg ml^{-1} of MLE depicted cell proliferation rate of 60.1% on day 3 and 50.8% on day 5 (Figure 1 *a*), whereas cell line incubated with 400 µg ml^{-1} of MLE depicted cell line proliferation rate of 56.1% and 20.1% on days 3 and 5 respectively (Figure 1 *b*). Thus, MLE demonstrated antiproliferative effect on MCF-7 cell line and inhibited MCF-7 cancer cell line with IC_{50} value of 1100 µg ml^{-1} (Supplementary Table 1). ALE of *G. nervosa* did not inhibit proliferation of MCF-7 cell line (Supplementary Figure 1).

Figure 2 depicts the effect of MLE on cell death of MCF-7 cell line. In comparison to the typical cell death observed in control cell line (no plant extract), the presence of 200 or 400 µg ml^{-1} of MLE exhibited higher cell death on day 3 in comparison to day 5. Addition of 200 µg ml^{-1} of MLE caused maximum cell death rate (52%) on day 3, compared to cell death rate (35%) observed on day 5 (Figure 2 *a*). Further, addition of 400 µg ml^{-1} of MLE demonstrated higher cell death rate (56%) on day 3 in comparison to cell death (31%) on day 5 (Figure 2 *b*).

Similar to *G. nervosa*, other plants from the Malvaceae family, e.g. *Sida rhombilifolia*, *Sida acuta*, *Sida cordifolium*, *Urena lobata* and *Viscum album* have been reported to inhibit cell proliferation of HepG-2 human liver cancer cell line[7]. Although extracts from *S. acuta* did not inhibit proliferation of MCF-7 cell line, they inhibited the proliferation of BT-20 and BT-549 cell lines (breast cancer cell lines) as well as PC-3 cell line (prostate cancer cell line)[8].

GC-MS analysis of MLE from *G. nervosa* revealed the presence of various phytochemicals (Table 1, Supplementary Figure 2). The ability of MLE of *G.*

nervosa to inhibit proliferation of MCF-7 cell line suggests the presence of compounds with antiproliferative property. α-Tocopherol (20.48%), a well-known antioxidant in MLE of *G. nervosa*, is already considered as an anticancer compound. Further, the antioxidative activity observed in the extracts of *Rheum officinale* Baill., *Sanguisorba officinalis* Linn. and *Paris polyphylla* Smith have been correlated with cytotoxicity against MCF-7 and AS49, adenoma carcinoma cell lines[9]. Furthermore, phytol (14.25%) detected in MLE has already been substantiated to demonstrate antiproliferative effect on hepatocellular cancer cell lines such as Huh-7 and HepG2. In addition, γ-sitosterol (4.48%) detected in MLE of *G. nervosa* has also been validated with antiproliferative activity against MCF-7 and lung carcinoma cell line A 549 (ref. 10). Thus, the concomitant presence of these three phytochemicals in MLE can be correlated to the antiproliferative activity observed against MCF-7 cell line. Additionally, the presence of α-linolenic acid (8.20%), an essential ω-3 fatty acid present in MLE is significant and has been associated with neuroprotective properties[11]. Similarly, *n*-hexadecanoic acid detected in MLE of *G. nervosa* is confirmed as a potent inhibitor of phospholipase A2 and would function as an anti-inflammatory agent[12].

Chronic inflammation has been commonly linked to the development of cancer. Furthermore, during the illness, patients suffer from neurological problems. Additional studies are being undertaken to evaluate the neuroprotective and anti-inflammatory properties of MLE. Thus the anti-inflammatory, neuroprotective and antiproliferative properties of MLE would make *G. nervosa* a prospective candidate for complementary therapy in cancer treatment.

1. Bandara, K. A., Kumar, V., Jacobsson, U. and Molleyres, L. P., *Phytochemistry*, 2000, **54**, 29–32.
2. Feng, S. X., Lin, L. D., Xu, H. H. and Wei, X. Y., *J. Asian Nat. Prod. Res*., 2008, **10**, 1155–1158.
3. Fan, H., Yang, G. Z., Zheng, T., Mei, Z. N., Liu, X. M., Chen, Y. and Chen, S., *Molecules*, 2010, **15**, 5547–5560.
4. Agarwal, G., Pradeep, P. V., Aggarwal, V, Yip, C. H. and Cheung, P. S., *World J. Surg.*, 2007, **31**, 1031–1040.
5. Mosmann, T., *J. Immunol. Methods*, 1983, **65**, 55–63.
6. Muthulakshmi, A., Jothibai, M. R. and Moham, V. R., *J. Appl. Pharm. Sci*., 2012, **2**, 69–74.
7. Pieme, C. A., Penlap, V. N., Ngogang, J. and Costache, M., *Environ. Toxicol. Pharmacol*., 2010, **29**, 223–228.
8. Fadeyi, S. A., Fadeyi, O. O., Adedeji, A. A., Okoro, C. and Myles, E. L., *BMC Complement. Altern. Med*., 2013, **13**, 79.
9. Li, W. Y., Chan, S. W., Guo, D. J. and Yu, P. H. F., *Pharm. Biol*., 2007, **45**, 541–546.
10. Sundarraj, S., Thangam, R., Sreevani, V., Kaveri, K., Gunasekaran, P., Achiraman, S. and Kannan, S., *J. Ethnopharmacol*., 2012, **141**, 803–809.
11. Blondeau, N., Lipsky, R. H., Bourourou, M., Duncan, M. W., Gorelick, P. B. and Marini, A. M., *Biomed. Res. Int*., 2015, 2015.
12. Aparna, V., Dileep, K. V., Mandal, P. K., Karthe, P., Sadasivan, C. and Haridas, M., *Chem. Biol. Drug Des*., 2012, **80**, 434–439.

Received 10 September 2016; revised accepted 23 June 2017

SURYA NANDAN MEENA[1]
SHIVANI SHARMA[2]
RITU KULSHRESHTHA[2]
MALAPATI K. JANARTHANAM[3]
SANJEEV C. GHADI[1,*]

[1]*Department of Biotechnology, and*
[3]*Department of Botany,*
Goa University,
Goa 403 206, India
[2]*Department of Biochemical Engineering and Biotechnology,*
Indian Institute of Technology,
New Delhi 110 016, India
**For correspondence.*
e-mail: saga@unigoa.ac.in

Distribution and conservation status of *Anthoceros macrosporus* Steph. (Anthocerotophyta) – an endemic and threatened hornwort of India

Anthoceros macrosporus Steph., an endemic and threatened hornwort which was known from its type specimen only from Borghat, Maharashtra, India has recently been re-discovered from Maharashtra and Gujarat from a second location in restricted pockets. Hence, it is proposed to designate the taxon as endangered and to be included in the Red List of Plants by the IUCN. Morphotaxonomic details of the recently collected plants have been studied.

The important hornwort family Anthocerotaceae is represented in India by two genera, viz. *Anthoceros* (Micheli) L. emend. Prosk. and *Folioceros* Bharadwaj. Nine valid species of the genus *Anthoceros* have been recognized from India[1]. *Anthoceros macrosporus* Steph. is a rarely occurring endemic species of India. It was instituted by Stephani[2] based on the specimens collected from Bor Ghat/Bhor Ghat, Khandala, Maharashtra in the Western Ghats in 1893. Later on it was also reported from Kodaikanal in Tamil Nadu[3], and Trimbakeshwar, Palghar and Matheran in Maharashtra[4], though the species could never be collected again from its type locality. Hence it is considered as extremely rare species[5] restricted to the Western Ghats, a prominent hotspot of the Indian subcontinent. The species has been rediscovered from the Kasara Ghat, Maharashtra and Saputara and Amba forest localities, Gujarat of the Western Ghats (Figure 1), in 2009 and 2011 respectively. The rediscovery of this taxon from a new locality in the vicinity of its original record is interesting and informative as the present finding facilitated the study of this species for the first time on fresh materials. The rarity of this taxon could also be explained from the fact that the workers who have revised the hornworts of India in the past decades could not get fresh samples of the species to describe *A. macrosporus*. They could