Indian Journal of Experimental Biology
Vol. 53, February 2015, pp. 98-103

Identification and characterization of a potent anticancer fraction from the leaf extracts of *Moringa oleifera* L.

Praveen T. Krishnamurthy[1]*, Ambalika Vardarajalu[2], Ashish Wadhwani[3] & Viral Patel[3]

[1]Department of Pharmacology; [2]Department of Phytopharmacy & Phytomedicine;
[3]Department of Biotechnology, JSS College of Pharmacy, Ootacamund, Tamil Nadu, India

Received 10 January 2014; revised 12 March 2014

Anticancer potential of *Moringa oleifera* L. extracts have been well established. However, there are no reports on the isolated molecules/fractions from these extracts which are responsible for the anticancer/cytotoxic activity. Thus, in the present study, we explored the same. The n-hexane, chloroform, ethyl acetate, methanol extracts of the *M. oleifera* leaves and 15 fractions (F1 to F15) of ethyl acetate extract were evaluated for their *in vitro* and *in vivo* anticancer activity using Hep-2 cell lines and Dalton's lymphoma ascites model in mice, respectively. Among the tested samples, the F1 fraction showed potential cytotoxic effect in Hep-2 cell lines with a CTC_{50} value of 12.5 ± 0.5 µg/ml. *In vivo* studies with the doses 5 and 10 mg/kg, p.o. demonstrated significant reduction in body weight and increased the mean survival time compared to the control group. These results were also comparable to the standard, 5-Fluorouracil, treated animals. We have also successfully isolated and characterized the anticancer fraction, F1 from the leaves of *M. oleifera* L.

Keywords: Antitumor activity, Benzoil tree, Bioassay guided fractionation, Cancer, Cytotoxicity, Dalton's lymphoma ascites, Drumstick tree, Hep-2 cell lines.

Cancer is one of the most common life-threatening diseases of mankind, causing as many as 7.6 million deaths in a year, 64% from the economically developing world. Although cancer incidence rates in the developing world are half of what has been observed in the developed world, the overall cancer mortality rates are generally similar[1]. Even a decade ago, India registered a steady increase in the Crude Incidence Rate (CIR) of all cancers affecting both men and women[2]. In 2010, India recorded 556400 national cancer deaths[3]. In US, cancer remains the second most common cause of death with an estimated deaths of 585720 in 2014[4]. The drugs currently used in the cancer chemotherapy have shown adverse effects such as nephrotoxicity, cardiotoxicity, peripheral neuropathies, etc[4-6]. On the other hand, natural products have contributed immensely in cancer chemotherapy. A diverse number of chemical structures have been isolated from the natural sources and some have lead to lead molecules, *viz*. Camptothecin, vincristine, vinblastine, taxol, podophyllotoxin, combretastatins, etc.[7-10]. Further, modifications on these leads have resulted in therapeutic agents including anticancer, such as Topotecan, irinotecan, taxotere, etoposide, teniposide, etc.[7-12]

Anticancer potentials of *Moringa oleifera* L. is not uncommon in folk medicine. Bose *et al*., discussed the role of this plant extracts in the treatment of epithelial ovarian cancer based on the presence of isothiocyanates group of phytoconstituents[13]. Earlier workers have reported the chemomodulatory effects of the hydroalcoholic extract of drumsticks against 7,12-dimethylbenz(a)anthracene induced skin papillomagenesis[14], and hepatic carcinogenesis in mice[15]. Budda *et al*., have shown the chemopreventive effects of freeze-dried pods against azoxymethane induced and dextran sodium sulfate promoted colon carcinogenesis in mice[16]. Sreelatha *et al*., have reported the antiproliferative and proapoptotic potentials of the *Moringa* leaf extracts using human tumor cell line[17]. Gismondi *et al*., have demonstrated the anticancer activity of the leaf fractions of this plant against B16F10 melanoma cells[18]. Although, thiocarbamates including niaziminin, isolated from the *Moringa* leaves possess antitumor promoter properties against teleocidin-B4 induced Epstein-Barr Virus activation in Raji Cells[19,20], there is no report on anticancer/cytotoxic compounds from these extracts.

In the present study, we have made an attempt to isolate and characterize the fractions responsible

*Correspondence:
Telefax: +91 423 2447135
E-mail: praveentk7812@gmail.com; praveentk7812@yahoo.co.in

for the anticancer activity of the leaves of *M. oleifera* by employing bioassay guided fractionation methodology.

Materials and Methods

Plant material and soxhlet extraction—The leaves of *M. oleifera* L. were collected from Coimbatore, Tamilnadu, India, and identified and authenticated by Dr. GVS Murthy, Joint Director, Botanical Survey of India, Coimbatore. A voucher specimen (TIFAC-01/2009) has been deposited for future reference at the Institution herbarium. The collected leaves (100 g) were subjected to successive soxhlet extraction using 750 ml each of n-hexane, chloroform, ethyl acetate and methanol (50%) for 24 h at 50-70 °C. The obtained extracts were concentrated under reduced pressure using rotary vacuum evaporator (Rotavapor® R-210/215, BÜCHI Labortechnik, AG).

Fractionation and standardization of ethyl acetate extract and F1—The extract (2 g) was packed into the silica gel (20 g) column by wet packing method. The column was eluted in a gradient manner using varying proportions of dichloromethane, methanol and ammonia (Fig. 1). A 20 ml fraction was collected in conical flasks and based on their TLC profiles they were combined and evaporated in rotary evaporator to obtain 15 fractions (F1 to F15).

Standardization of ethyl acetate extract and F1—The standardization was done using HPTLC finger printing analysis using CAMAG HPTLC analyzer (CAMAG, Muttenz, Switzerland). Five microlitre of each sample prepared in methanol (1 mg/ml) was placed on precoated silica gel TLC plate (254F, Merck, Darmstadt, Germany) using CAMAG Linomat IV applicator. TLC was developed in CAMAG twin trough development chamber using chloroform: methanol (9:1) as mobile phase. After development, the TLC plate was dried and scanned using CAMAG HPTLC Scanner III, integrated with WIN CATS software (version 4.06) and the peaks were recorded at 366 nm. In addition, these extracts were also subjected to qualitative phytochemical analysis to determine the presence of various categories of phytoconstituents.

In vitro anticancer activity—Cell lines used in the present study were obtained from National Centre for Cell Science (NCCS), Pune, India. The Dalton's Lymphoma Ascites (DLA), Vero and Human Epidermoid Cancer (Hep-2) cells were maintained in RPMI-1640 medium supplemented with 10% FBS, glutamine (2 mM), penicillin (100 units/ml) and streptomycin (100 µg/ml). The cells were cultured at 37 °C in a humidified 5% CO_2 incubator.

The *in vitro* cytotoxicity of all the extracts and fractions were estimated against Vero cell cultures as

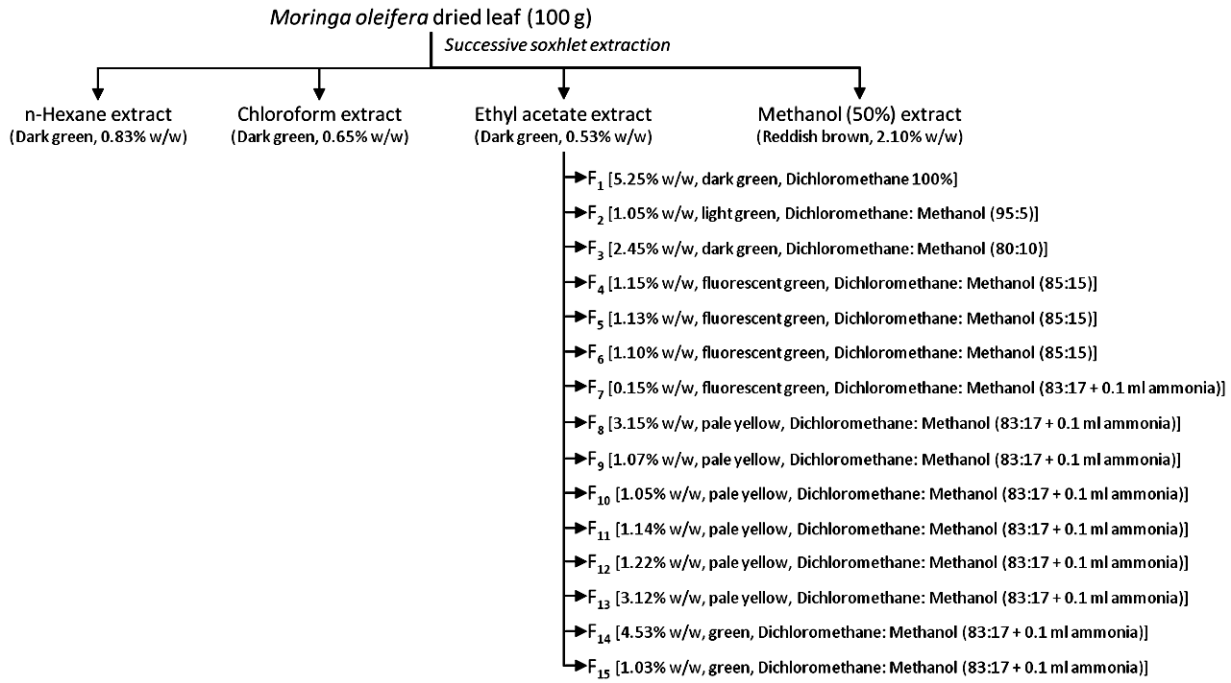

Fig. 1—Extraction and isolation scheme for the leaves of *Moringa oleifera* L.

per the standard procedures described in the below mentioned assay for Hep-2 cell lines. The *in vitro* anticancer activity of the extracts and fractions was assessed against Hep-2 cell lines[21]. The monolayer culture of Hep-2 cell line was trypsinized and the cell count was adjusted to 1.0×10^5 cells/ml using medium containing 10% new born calf serum. The diluted cell suspension (0.1 ml) was added to each well of the 96-well microplates. The test wells were added with various concentrations (100 µl) of test samples and the control wells received media (100 µl). The plates were then incubated at 37 °C for three days in 5% CO_2 atmosphere. Morphological changes were recorded daily for both control and treated wells to assess the changes such as aggregation, shrinking, membrane blebbing, ballooning, etc. After 72 h, the cultures were fixed with trichloroacetic acid (25 µl, 10% w/v) and stained for 30 min with sulforhodamine B (0.4% w/v) dissolved in acetic acid (1% v/v). Unbound dye was removed by four washes with acetic acid (1% v/v), and protein-bound dye was extracted with 10 mM unbuffered Tris base [tris (hydroxymethyl)aminomethane] for determination of optical density at 540 nm. The percentage cell viability (CV) was calculated using the following formula:

$$CV = \frac{\text{Absorbance of test}}{\text{Absorbance of control}} \times 100$$

The concentration required to kill 50% of the cells (CTC_{50}) was calculated from the regression line obtained by plotting percentage CV *vs*. concentration.

In vivo studies

Animals—Adult Swiss albino mice of either sex were used for acute oral toxicity and *in vivo* anticancer studies. Prior approval of the Institutional Animal Ethics Committee (Approval no. JSSCP/ IAEC/M.Pharm/Phytopharm/08/2009-2010) was obtained for conducting this study. The animals were housed in polypropylene cages in a controlled environment (Temperature 23±2 °C and 12 h L:D cycle) with standard laboratory diet (M/s. Amruth Labs Private Ltd., Bangalore) and water *ad libitum* in the animal house of the institute.

Acute oral toxicity of F1—Acute oral toxicity of F1 was carried out as per the OECD guideline for testing of the chemicals[22]. A limit test at a dose of 2000 mg/kg, p.o., was carried out using 3 female mice per step. After extract administration, all the animals were observed for clinical signs and mortality at 0, 0.5, 1, 2 and 4 h, and thereafter daily for a period of 14 days. During the study period, weekly body weight of all the animals was recorded. At the end of the study, all the survived animals were culled by deep ether anaesthesia and subjected to gross necropsy analysis.

In vivo anticancer activity—Based on the results of *in vitro* studies, the F1 was chosen for the *in vivo* studies. The anticancer activity was assessed using DLA model in mice[23]. Thirty male mice were divided into 5 groups of 6 each. All the animals were injected intraperitoneally with 1×10^6 DLA cells. Group-1 and Group-2 animals received vehicle (0.2% w/v Tween-80, 10 ml/kg, p.o.) and served as a normal and test control, respectively. Group-3 animals received 5-fluorouracil (10 mg/kg, p.o.). Group 4 and Group 5 animals received F1 at a dose of 5 and 10 mg/kg, p.o., respectively. The dose levels of F1 were selected based on the *in vitro* results and also by considering the acute oral toxicity results. The treatment was started 24 h after tumor inoculation and continued for a period of 10 days. During the study period, all the animals were observed for clinical signs and mortality. The body weights of all the animals were recorded every 3 days once during the study period. The percentage increase in mean survival time (%MST) was calculated using the following formula:

$$\%MST = \left(1 - \frac{T}{C}\right) \times 100$$

T= Mean survival time of treated group; C= Mean survival time of control group.

Statistical analysis—The data were represented as mean ± SD and analyzed by one-way ANOVA followed by Dunnett's multiple comparison tests using Prism software (version 4). *P* values ≤0.05 were considered significant.

Results

Extraction and fractionation—The yield of n-hexane, chloroform, ethyl acetate, methanol extracts of *Moringa oleifera* leaves and ethyl acetate fractions are given in Fig. 1. Among the extracts, methanol extract was obtained at highest yield (2.10 % w/w) and among the fractions; F1 with an yield of 5.25% w/w.

Standardization of ethyl acetate extract and F1—The HPTLC standardization of ethyl acetate extract and F1 showed 13 and 5 well resolved peaks, respectively (Fig. 2). The qualitative phytochemical

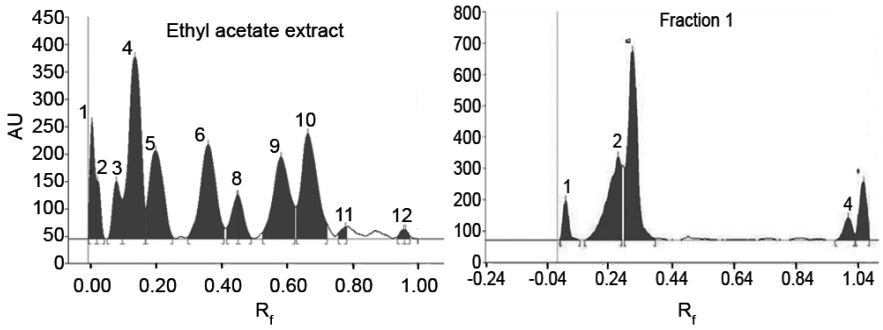

Fig. 2—HPTLC finger printing of ethyl acetate extract and F1.

Table 1—*In vitro* anticancer activity of extracts and fractions of *Moringa Oleifera* L. in Hep-2 cell lines. Values are mean ± SD, n=3.

Name of the sample	IC_{50} (µg/ml)
n- Hexane extract	180.6 ± 10.8
Chloroform extract	190.2 ± 15.8
Methanol (50%) extract	170.1 ± 19.8
Ethyl acetate extract	40.2 ± 1.8
Fraction 1	12.5 ± 0.5
Fraction 2	74.5 ± 5.4
Fraction 3	39.4 ± 2.3
Fraction 4	100.1 ± 1.9
Fraction 5	64.3 ± 1.4
Fraction 6	73.2 ± 1.8
Fraction 7	71.4 ± 1.7
Fraction 8	79.4 ± 3.6
Fraction 9	111.2 ± 5.1
Fraction 10	121.4 ± 7.1
Fraction 11	81.2 ± 3.8
Fraction 12	91.3 ± 5.8
Fraction 13	130.5 ±7.1
Fraction 14	88.3 ± 3.8
Fraction 15	71.3 ± 2.8

results revealed the presence of steroids, flavonoids and phenolic compounds in ethyl acetate extracts, whereas, the F1 showed the presence of steroids and phenolic compounds.

In vitro anticancer activity—In the *in vitro* cytotoxicity studies with the Vero cell lines all the extracts and fractions showed a CTC_{50} > 300 µg/ml. A concentration range of 4-250 µg/ml was, therefore, employed for *in vitro* anticancer studies against Hep2 cell lines. Among the extracts, the ethyl acetate extract showed considerable anticancer activity against Hep-2 cell lines and, therefore, selected for further fractionation. Among the fractions, the F1 showed a potent anticancer activity, whereas the other fractions obtained showed mild to moderate activity (Table 1, Fig. 3).

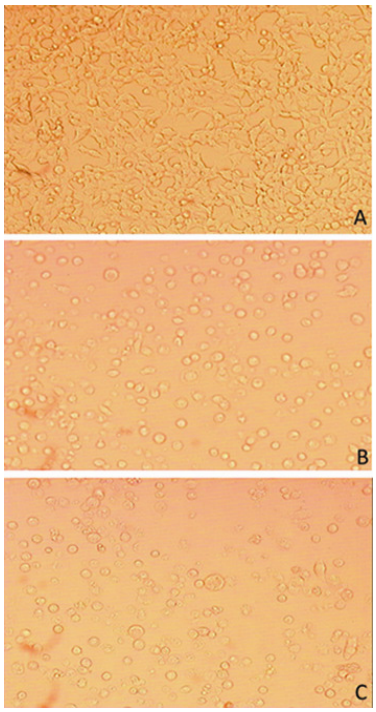

Fig. 3—Morphological changes of Hep-2 cells. **(**A) Normal cells showing the normal morphological features; (B and C) Treated with ethyl acetate extract (31.25 µg/ ml) and F1 (7.8 µg/ ml), respectively, showing the loss of monolayer.

Acute oral toxicity of F1—In the acute oral toxicity study, mice treated with F1 (2000 mg/kg, p.o.) showed no abnormal clinical signs and mortality. All the animals gained 4-8 g of body weight during the study period and the gross necropsy carried out on these animals reveal no gross lesions. Based on these results the acute oral toxicity of F1 was, therefore, classified as GHS category 5 (LD_{50} >2000 mg/kg).

In vivo anticancer activity—The F1 in the in the *in vivo* anticancer studies showed a dose dependent activity against DLA induced peritoneal ascites in mice. The F1, dosed at 5 and 10 mg/kg, p.o., in mice, showed a significant (*P* <0.05) lesser body weight

Table 2—Effect of F1 on DLA induced changes in the mice body weight

Values are mean ± SD, n=6

Group	Group1 (Normal)	Group 2 (Control)	Group 3	Group 4	Group 5
Treatment	Tween (0.2%) 10 ml/kg, p.o.	Tween (0.2%) 10 ml/kg, p.o.	5-Flurouracil, 10 mg/kg, i.p.	Fraction 1, 5 mg/kg, p.o.	Fraction 1, 10 mg/kg, p.o.
Day 0	25.8 ± 4.0	27.2 ± 3.1	25.7 ± 3.0	28.5 ± 4.4	27.7 ± 2.9
Day 3	25.0 ± 2.8	28.5 ± 3.6	26.2 ± 3.4	27.5 ± 3.8	28.8 ± 1.7
Day 6	25.0 ± 2.4	35.5 ± 2.0$^{\#}$	27.2 ± 4.1*	28.5 ± 3.3*	28.8 ± 3.9*
Day 9	26.7 ± 2.9	42.7 ± 1.8$^{\#}$	30.0 ± 1.1*	37.2 ± 2.6*	35.0 ± 2.1*
Day 12	27.3 ± 2.5	43.0 ± 1.7$^{\#}$	32.2 ± 2.8*	42.5 ± 2.1	38.0 ± 2.4*
Day 15	27.8 ± 3.8	48.5 ± 1.9$^{\#}$	34.3 ± 2.0*	46.8 ± 1.9	38.7 ± 2.3*
Day 18	27.2 ± 2.7	54.0 ± 1.7$^{\#}$	37.2 ± 1.9*	45.7 ± 2.1*	37.8 ± 1.8*

$^{\#}P$ <0.05 when compared to Normal, *P <0.05 when compared to control

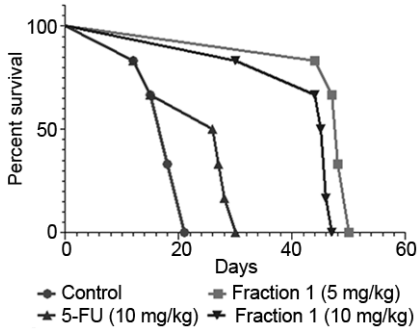

Fig. 4—Effect of F1 on survival time of mice against DLA induced ascites

and a significant increase in the mean survival time when compared to the vehicle treated control mice (Table 2, Fig. 4). These results were also comparable to the standard, 5-Fluorouracil treated animals.

Discussion

Bioassay guided fractionation played an important role in the isolation of active compounds/fractions from natural products[24-29]. In the present study, we have also employed bioassay guided fractionation to isolate the anticancer compounds/fractions from the leaves. Although no anticancer compounds could be isolated from the leaves, we could successfully isolate and characterize a potent anticancer fraction, F1. The F1 has demonstrated promising anticancer activities in both *in vitro* and *in vivo* studies and its activity is comparable to the standard 5-fluorouracil (Tables 1 & 2, Figs. 3 & 4). The qualitative phytochemical study results revealed the presence of steroids and phenolic compounds in F1. Many natural compounds belonging to this class have been reported to possess cytotoxic and anticancer potentials[30-33]. The cytotoxic and anticancer potentials of F1 is, therefore, may be attributed to the presence of this class of compounds.

Conclusion

In the present work, we have successfully isolated and characterized the fraction, F1, present in the leaves of *M. oleifera* L. possibly responsible for its anticancer activity and tested its cytotoxic/anticancer potential.

References

1 Ahmedin Jemal, Freddie Bray, Melissa M. Center, Jacques Ferlay, Elizabeth Ward & David Forman, Global Cancer Statistics, *CA Cancer J Clin* 61 (2011) 69

2 Pal SK & Mittal B, Improving cancer care in India: prospects and challenges, *Asian Pac J Cancer Prev*, 5 (2004) 226.

3 Dikshit R, Gupta PC, Ramasundarahettige C, Gajalakshmi V, Aleksandrowicz L, Badwe R, Kumar R, Roy S, Suraweera W, Bray F, Mallath M, Singh PK, Sinha DN, Shet AS, Gelband H, Jha P & Million, Death Study C, Cancer mortality in India: a nationally representative survey, *Lancet*, 379 (2012) 1807.

4 Cancer Facts & Figures 2014, American Cancer Society, http://www.cancer.org/research/ cancerfactsstatistics/cancerfactsfigures2014/ as accessed on 27 December 2014.

5 Merz B & Hager T, Defusing adverse effects of anticancer drugs, *JAMA*, 250 (1983) 459.

6 Grosso F & D'incalci M, Problems in dealing with very rare adverse effects of new anticancer drugs: the example of trabectedin, *Tumori*, 97 (2011) 256.

7 Monneret C, Current impact of natural products in the discovery of anticancer drugs, *Ann Pharm Fr*, 68 (2010) 218.

8 Coseri S, Natural products and their analogues as efficient anticancer drugs, *Mini Rev Med Chem*, 9 (2009) 560.

9 Sak K, Chemotherapy and dietary phytochemical agents, *Chemother Res Pract*, 2012 (2012) 282570.

10 Cretu E, Trifan A, Vasincu A & Miron A, Plant-derived anticancer agents - curcumin in cancer prevention and treatment, *Rev Med Chir Soc Med Nat Iasi*, 116 (2012) 1223.

11 Tsubura A, Lai YC, Kuwata M, Uehara N & Yoshizawa K, Anticancer effects of garlic and garlic-derived compounds

for breast cancer control, *Anticancer Agents Med Chem*, 11 (2011) 249.

12 Saklani A & Kutty SK, Plant-derived compounds in clinical trials, *Drug Discov Today*, 13 (2008) 161.

13 Bose CK, Possible role of *Moringa oleifera* Lam. root in epithelial ovarian cancer, *Med Gen Med*, 9 (2007) 26.

14 Bharali R, Tabassum J & Azad MR, Chemomodulatory effect of *Moringa oleifera* Lam., on hepatic carcinogen metabolising enzymes, antioxidant parameters and skin papillomagenesis in mice, *Asian Pac J Cancer Prev*, 4 (2003) 131.

15 Sharma V, Paliwal R, Janmeda P & Sharma S, Chemopreventive efficacy of *Moringa oleifera* pods against 7, 12-dimethylbenz[a]anthracene induced hepatic carcinogenesis in mice, *Asian Pac J Cancer Prev*, 13 (2012) 2563.

16 Budda S, Butryee C, Tuntipopipat S, Rungsipipat A, Wangnaithum S, Lee JS & Kupradinun P, Suppressive effects of Moringa oleifera Lam. pod against mouse colon carcinogenesis induced by azoxymethane and dextran sodium sulfate, *Asian Pac J Cancer Prev*, 12 (2011) 3221.

17 Sreelatha S, Jeyachitra A & Padma PR, Antiproliferation and induction of apoptosis by *Moringa oleifera* leaf extract on human cancer cells, *Food Chem Toxicol*, 49 (2011) 1270.

18 Gismondi A, Canuti L, Impei S, Di Marco G, Kenzo M, Colizzi V & Canini A, Antioxidant extracts of African medicinal plants induce cell cycle arrest and differentiation in B16F10 melanoma cells, *Int J Oncol*, (2013)

19 Faizi S, Siddiqui BS, Saleem R, Siddiqui S, Aftab K & Gilani AH, Fully acetylated carbamate and hypotensive thiocarbamate glycosides from *Moringa oleifera*, *Phytochemistry*, 38 (1995) 957.

20 Murakami A, Kitazono Y, Jiwajinda S, Koshimizu K & Ohigashi H, Niaziminin, a thiocarbamate from the leaves of *Moringa oleifera*, holds a strict structural requirement for inhibition of tumor-promoter-induced Epstein-Barr virus activation, *Planta Med*, 64 (1998) 319.

21 Skehan P, Storeng R, Scudiero D, Monks A, Mcmahon J, Vistica D, Warren JT, Bokesch H, Kenney S & Boyd MR, New colorimetric cytotoxicity assay for anticancer-drug screening, *J Natl Cancer Inst*, 82 (1990) 1107.

22 Schlede E, Mischke U, Roll R & Kayser D, A national validation study of the acute-toxic-class method--an alternative to the LD50 test, *Arch Toxicol*, 66 (1992) 455.

23 Natesan S, Badami S, Dongre SH & Godavarthi A, Antitumor activity and antioxidant status of the methanol extract of *Careya arborea* bark against Dalton's lymphoma ascites-induced ascitic and solid tumor in mice, *J Pharmacol Sci*, 103 (2007) 12.

24 Weller MG, A unifying review of bioassay-guided fractionation, effect-directed analysis and related techniques, *Sensors* (Basel), 12 (2012) 9181.

25 Vasskog T, Andersen JH, Hansen E & Svenson J, Characterization and cytotoxicity studies of the rare 21:4 n-7 acid and other polyunsaturated fatty acids from the marine opisthobranch *Scaphander lignarius*, isolated using bioassay guided fractionation, *Mar Drugs*, 10 (2012) 2676.

26 Machado FB, Yamamoto RE, Zanoli K, Nocchi SR, Novello CR, Schuquel IT, Sakuragui CM, Luftmann H, Ueda-Nakamura T, Nakamura CV & De Mello JC, Evaluation of the antiproliferative activity of the leaves from *Arctium lappa* by a bioassay-guided fractionation, *Molecules*, 17 (2012) 1852.

27 Tocci N, D'auria FD, Simonetti G, Panella S, Palamara AT, Debrassi A, Rodrigues CA, Filho VC, Sciubba F & Pasqua G, Bioassay-guided fractionation of extracts from *Hypericum perforatum in vitro* roots treated with carboxymethylchitosans and determination of antifungal activity against human fungal pathogens, *Plant Physiol Biochem*, 70C (2013) 342.

28 Tayarani-Najaran Z, Mousavi SH, Tajfard F, Asili J, Soltani S, Hatamipour M & Emami SA, Cytotoxic and apoptogenic properties of three isolated diterpenoids from *Salvia chorassanica* through bioassay-guided fractionation, *Food Chem Toxicol*, 57 (2013) 346.

29 Frank FM, Ulloa J, Cazorla S I, Maravilla G, Malchiodi EL, Grau A, Martino V, Catalan C & Muschietti LV, Trypanocidal Activity of *Smallanthus sonchifolius*: Identification of Active Sesquiterpene Lactones by Bioassay-Guided Fractionation, *Evid Based Complement Alternat Med*, 2013 (2013) 627898.

30 Phi TD, Pham VC, Thi Mai HD, Litaudon M, Gueritte F, Nguyen VH & Chau VM, Cytotoxic steroidal alkaloids from *Kibatalia laurifolia*, *J Nat Prod*, 74 (2011) 1236.

31 Zhang C, Feng S, Zhang L & Ren Z, A new cytotoxic steroidal saponin from the rhizomes and roots of *Smilax scobinicaulis*, *Nat Prod Res*, 27 (2013) 1255.

32 Al-Sayed E, Martiskainen O, Bobrowska-Hagerstrand M, Sinkkonen J, Tornquist K, Pihlaja K, Ayoub N & Singab AN, Phenolic compounds from *Eucalyptus gomphocephala* with potential cytotoxic and antioxidant activities, *Nat Prod Commun*, 5 (2010) 1639.

33 Shen S, Ding X, Ouyang MA, Wu ZJ & Xie LH, A new phenolic glycoside and cytotoxic constituents from *Celosia argentea*, *J Asian Nat Prod Res*, 12 (2010) 821.