In vitro antioxidant potential and antiproliferative activity of Aesculus indica seed extract against hepatocellular carcinoma cell line

Article *in* Pakistan Journal of Pharmaceutical Sciences · January 2021

DOI: 10.36721/PJPS.2021.34.1.SUP.291-300.1

CITATIONS
2

READS
304

8 authors, including:

Sadia Perveen
Università degli Studi di Torino
7 PUBLICATIONS **32** CITATIONS
SEE PROFILE

Khalid Hussain
University of Lahore
154 PUBLICATIONS **1,417** CITATIONS
SEE PROFILE

Shamaila Azam
University of the Punjab
2 PUBLICATIONS **3** CITATIONS
SEE PROFILE

Ayisha Shaukat
University of the Punjab
14 PUBLICATIONS **21** CITATIONS
SEE PROFILE

doi.org/10.36721/PJPS.2021.34.1.SUP.291-300.1

In vitro antioxidant potential and antiproliferative activity of *Aesculus indica* seed extract against hepatocellular carcinoma cell line

Hina Shahbaz[1], Abida Latif[1], Sadia Perveen[2], Khalid Hussain[1], Shamaila Azam[1], Ayisha Shaukat[1], Nadeem Irfan Bukari[1] and Asima Tayyeb[2]*

[1]University College of Pharmacy, University of the Punjab, Lahore, Pakistan
[2]School of Biological Sciences, University of the Punjab, Quaid-e-Azam Campus, Lahore, Pakistan

Abstract: Therapeutic secondary metabolites have gained immense attention in recent years due to their effective medicinal properties. *Aesculus indica* is a medicinally important plant being traditionally used for various ailments. The present study aimed to determine the antioxidant and antiproliferative activities of seeds of *A. indica.* The crude methanolic seed extract was prepared and subjected to sequential fractionation in increasing polarity. The extract and its fractions were investigated for antioxidant activities using various *in vitro* assays. Further, the extract along with its potential antioxidant fractions were analyzed for their cytotoxic activity against HepG2, human hepatocyte carcinoma cells through bioassays. The results showed highly significant antioxidant potential of methanolic extract of *A. indica* seeds and two of its fractions prepared with chloroform and ethyl acetate. The studies on hepatocyte carcinoma cells further revealed that the extract and two of its potential antioxidant fractions significantly induced cytotoxicity and inhibited migration, proliferation, clonogenicity and 3D growth of HepG2 cells. It is therefore, concluded that *A. indica* possess significant antioxidant and cytotoxic potential against HepG2 cells and with further research can be proposed for therapeutic interventions.

Keywords: *Aesculus indica*, antioxidant, bioassays, antiproliferative, HepG2 cell line.

INTRODUCTION

Therapeutic natural products obtained from animals, plants and minerals are the source of drugs since the beginning of humankind (Newman, 2018; Rates, 2001). Numerous antioxidant compounds have been isolated from natural resources including medicinal plants, fungi and bacteria and are used in various cosmetic, nutraceutical and therapeutic applications (Chandra *et al.*, 2020; Oroian & Escriche, 2015). Thousands of plant species have been investigated for their medicinal properties. The drug discovery from plants is related to their ethno-pharmacological usage and most of the plant derived drugs are also associated to their traditional medicinal use from long time ago (Aslam & Ahmad, 2016; Fabricant & Farnsworth, 2001). A large number of presently used plants derived medicines were identified from the effective and remedial traditional use of that plant and were later on isolated and synthesized on a mass scale. Many cytotoxic drugs have been identified from plant sources like vincristine, irinotecan, etoposide and paclitaxel. Similarly, dactinomycin, bleomycin and doxorubicin are anti-cancer drugs derived from microbial sources (Da Rocha *et al.*, 2001; Shah *et al.*, 2013).

Aesculus genus belongs to the Sapindaceae family. This genus comprises of 12 species of deciduous trees and shrubs found in Eastern Asia, Europe and various parts of North America (Z. Zhang *et al.*, 2010). *Aesculus hippocastanum* and *Aesculus chinensis* are medicinally important plants and have been cultivated as medicinal crop for the development of Standardized Therapeutic Extracts (STE) (Ramirez-Duron *et al.*, 2007). *Aesculus indica* is commonly known as Indian horse chestnut and locally called as bandkhor, jawaz or jaumuqadam (Guleria & Vasishth, 2009; Sood *et al.*, 2015). The plant parts have been used traditionally for rheumatism, skin diseases, jaundice cough and vein complaints (Bibi *et al.*, 2012; Haq *et al.*, 2011). The various parts of *A. indica* are rich in therapeutic secondary metabolites. The leaves and seeds of *A. indica* have been reported for the presence of carbohydrates, proteins, long fatty chain compounds, flavonoids, triterpenoids, carotenoids, steroids, saponins, terpenes and different mineral contents (Firdoos *et al.*, 2018; Mishra *et al.*, 2018). The leaves have been found to be effective in exhibiting cytotoxic potential against breast adenocarcinoma cells (Bibi *et al.*, 2012). The leaves have also shown significant stimulation of cell mediated immunity in *in vivo* models. Furthermore, the leaf extract of this plant also exhibits significant antioxidant activity (Chakraborthy, 2009). However, the antioxidant and cytotoxic studies of *A. indica* seeds and its fractions are not yet documented. The study was conducted to determine the antioxidant and cytotoxic potential of *A. indica* seeds. Furthermore, antiproliferative potential of *A. indica* was also investigated against HepG2 hepatic carcinoma cells through various bioassays.

**Corresponding author:* e-mail: asmaalam11@hotmail.com

MATERIALS AND METHODS

Preparation of seed extract and fractions

A. indica seeds were purchased from local market and identified by Dr. Zaheer ud din, Department of Botany Government College University, Pakistan and were given voucher number GC. Herb. Bot. 3632. The methanolic extract (ME Ext) of the powdered seeds was prepared using Soxhlet's apparatus. The solid crude extract was obtained by evaporating the solvent in rotary evaporator Heidolph Laborota L-4002. The ME Ext was fractionated in separating funnel by suspending the 100 g extract in 150ml of water dividedly and then partitioned to prepare petroleum ether (PE Fr.), chloroform (CH Fr.), ethyl acetate (ETAc Fr.), n-butanol (n-BUT Fr.) and aqueous (WA Fr.) fractions followed by rotary evaporation.

In vitro antioxidant assays

Antioxidant activity of ME Ext and its fractions was evaluated through following in vitro assays:

1,1-diphenyl-2-picryl-hydrazyl (DPPH) Assay

Varying concentrations (100-1000µg/ml) of samples were made in methanol. In a test tube, sample (1ml), 0.1mM DPPH (1ml) and methanol (1ml) were mixed followed by 10 minutes incubation in dark at 37°C. DPPH solution was taken as control (Patel *et al.*, 2010). The absorbance (A) was measured at 517nm. Following formula was used to evaluate percentage inhibition of radicals.

$$\% \text{ Inhibition} = \frac{(A_{\text{Control}} - A_{\text{Sample}})}{A_{\text{Control}}} \times 100$$

Ferric reducing power assay

The samples were prepared in concentration range 20-200 µg/ml in methanol. The reaction mixture was prepared in a test tube by taking 0.5ml of diluted sample along with 2.5ml of each phosphate buffer (pH 6.6) and potassium ferricyanide (1% w/w). After incubation for about 30 minutes in water bath at 50°C, 2.5ml of trichloroacetic acid (10% w/v) was added and centrifuged for 10 mins. The supernatant (2.5ml) taken was diluted with distilled water and ferric chloride (0.1% w/w). After mixing it thoroughly the absorbance was measured at 700nm (Chu *et al.*, 2000). Trolox was used as a standard.

Total antioxidant activity

The sample (0.1ml) was mixed with phosphomolybdate reagent (3ml) and incubated for 90 min in water bath at 95°C. The reaction mixture was cooled at room temperature and the absorbance was measured at 695nm (Abdel-Hameed *et al.*, 2012). The antioxidant activity of the samples was expressed as ascorbic acid equivalent.

Anti-proliferative Activity against HepG2 Cells

The ME Ext and its fractions exhibiting the highest antioxidant activity were evaluated further for antiproliferative activity against HepG2 cells.

Cell Culture

HepG2 cell line (Human Hepatocyte Carcinoma) was obtained from cell culture stock of School of Biological Sciences, University of the Punjab, Pakistan. The cells were cultured in DMEM (Dubecco's Modified Eagle Medium) prepared with 10% FBS (Fetal Bovine Serum) and 1% penicillin-streptomycin under humidified controlled atmosphere at 37°C with 5% CO_2 in cell culture incubator. All the cell culture reagents were obtained from Gibco and Sigma.

Cytotoxicity Assay

The cytotoxic effects were determined by the MTT assay on HepG2 cells as described previously (Kanwal *et al.*, 2019). The cells were cultured in a 96-well plate at a density of 10,000 cells/well. After 24h, *A. indica* ME Ext and its fractions in varying concentrations (0-500 µg) were subjected to the cells compared to control with equal volume of DMEM. After 72h, in each well, 10µl of MTT (3-(4, 5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide) solution (5mg/ml) was added followed solubilization of formazan crystals with DMSO solution (100µl). The absorbance was determined at 570nm with ELISA plate reader for the experimental wells with respect to corresponding control wells.

Scratch Assay

The scratch assay was performed to evaluate the cell migration as described by Mandal *et al*., 2011. Cells were seeded about 2 $x10^5$ cells/well of a six well tissue culture plate. A micropipette tip was used to generate a scratch in cells grown to 95% confluency. The cells were treated with *A. indica* ME Ext and its fractions at their respective LC_{50} doses for 72h separately, and microscopy was performed to visualize and document the changes in scratch.

Sphere Formation Assay

Soft agar assay was performed by slightly modified method as described by Sebolt-Leopold *et al*., 1999. 96 well cell culture plates coated with 1.5% agarose and used to seed 3000 cells per well in DMEM media containing LC_{50} concentration of the *A. indica* ME extract and its fractions and incubated for 8 days. CellSens software in bright field of Olympus florescence microscope was used to study the morphology and measure the size of the spheroids.

Colony Formation Assay

The method described by Hirsch *et al*., 2009 was used to evaluate colony formation potential. Briefly, cells were seeded at a density of 500 cells/well of a six well plate, treated with *A. indica* extract and its fractions separately, and incubated under cell culture conditions for one week. Colonies were fixed with methanol and acetic acid (3:1), stained with 0.5% crystal violet solution for 15 minutes and observed under the bright field of inverted microscope.

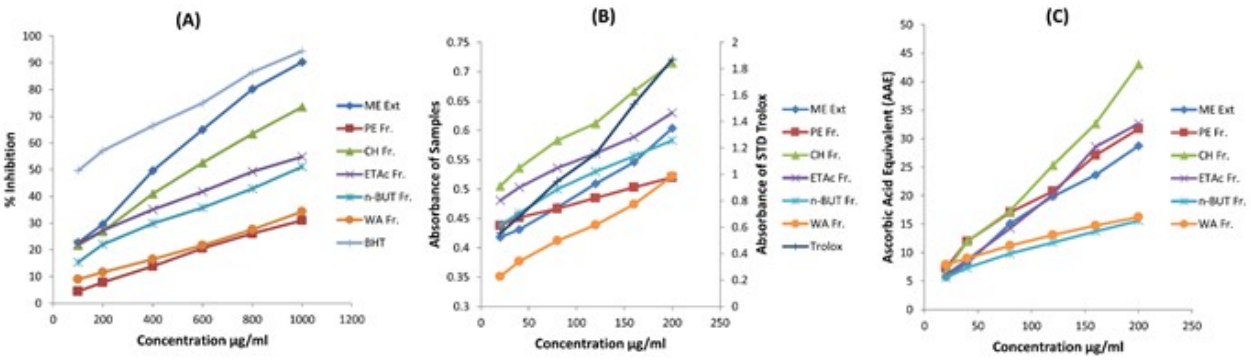

Fig. 1: (A) DPPH Radical Scavenging Activity of *Aesculus indica* (B) Ferric Reducing Power of *Aesculus indica* (C) Total Antioxidant Assay of *Aesculus indica*

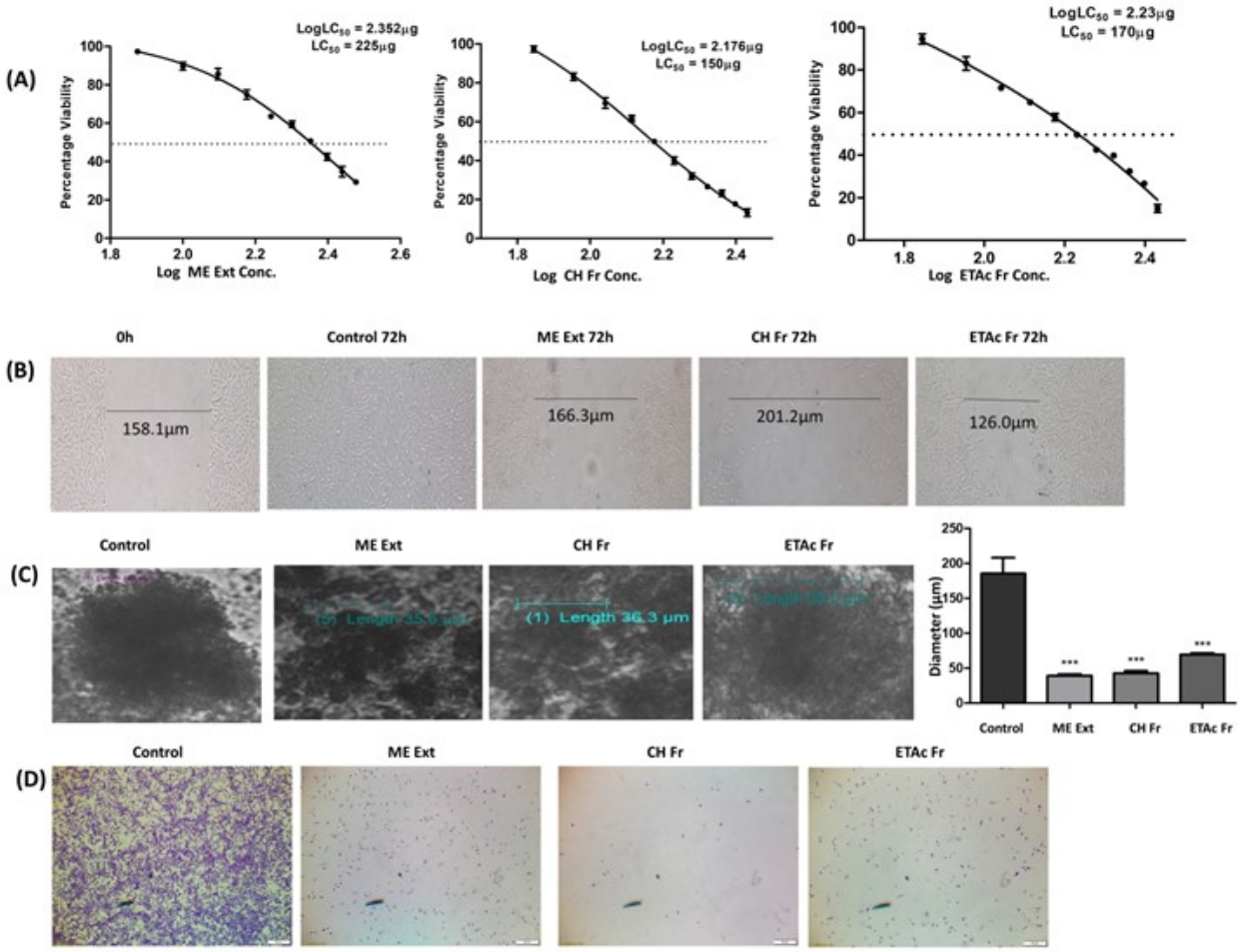

Fig. 2: (A) Cell Viability Assay (B) Wound Healing Assay (C) Spheroid Formation Assay (D) Colony Formation Assay (*P<0.05, **P<0.01, ***P<0.001).

STATISTICAL ANALYSIS

The statistical analysis was performed with Microsoft excel program and GraphPad Prism Version 8.4.2. The tests were conducted in triplicates and the data was presented in mean values and the standard error of the mean (Mean ± S.E). To analyze data, one-way ANOVA and multiple comparisons of data with the control was accomplished. P<0.05, P<0.01& P<0.001 was considered as significant.

RESULTS

A. indica possesses strong antioxidant capacity

A. indica ME Ext and its fractions possess potent antioxidant capacity in a concentration dependent manner. The DPPH assay depicted good radical scavenging activity. Butylated hydroxy toulene (BHT) was used as a standard and it showed the highest percentage inhibition. This activity was decreased in the order, BHT >ME Ext > CH Fr. >ETAc Fr >n-BUT Fr. >WA Fr. >PE Fr (fig. 1A). Ferric Reducing Power Assay revealed that that all the samples exhibited the electron donating capacity and thus possessing strong antioxidant capacity. ME Ext represented good antioxidant activity and its CH Fr. showed the highest reducing than all other fractions followed by ETAc Fr (fig. 1B). Total Antioxidant Assay also indicated strong antioxidant activity in all the samples. The maximum activity was observed with Ch Fr. followed by ETAc Fr. and PE Fr. The ascorbic acid equivalent of n-BUT Fr and WA Fr. was low and differs insignificantly from each other (fig. 1C).

A. indica inhibit growth, proliferation and migration potential of liver cancer cells

The effectiveness of ME Ext and its strong antioxidant fractions (CH Fr. and ETAc Fr.) against HepG2 cells was determined with LC_{50} values using the MTT assay data. Cell viability was reduced in a dose dependent manner as compared to control. The LC_{50} values for ME Ext, Ch Fr. and ETAc Fr. were 225µg, 150µg and 170µg, respectively (fig. 2A). These findings clearly indicated the efficacy of Ch Fr. against HepG2 cells as compared to the ETAc Fr. and ME Ext. The migratory property of the HepG2 cells in the presence of ME Ext and its CH Fr. was significantly reduced. In treated cells, the gap area that was unfilled in between the scratches was larger as compared to the control (fig. 2B). ME Ext and its fraction also inhibited the 3D growth potential of HepG2 cells. It was observed that the treated cells showed small number and average diameter of the spheres relative the control (fig. 2C). Colony formation assay indicated the inhibitory action of *A. indica* towards the clonogenicity potential. It was observed that the number of colonies formed in cells treated with CH Fr. was reduced to the maximum followed by ETAc Fr. and ME Ext when compared with the control (fig. 2D).

DISCUSSION

A. indica is a traditionally used medicinal plant well known to cure various ailments. However, molecular mechanisms responsible for the plant's significant medicinal value have not yet studied. Many reports have suggested the utilization of plants rich in antioxidants can reduce the risk of cancer and other diseases (Farinati *et al.*, 2007; Navaneetha Krishnan *et al.*, 2019; Prasad *et al.*, 2017). In the present study, ME Ext of *A. indica* and its Ch Fr. and ETAc Fr. have exhibited significant antioxidant capacity through various antioxidant assays. Uncontrolled proliferation is one important hall mark of cancer cells and hepatocellular carcinoma cell line, HepG2 is well known model with strong growth potential. The present study highlighted *A. indica* as an anticancer agent especially by reducing the proliferation potential of HepG2 cells. A number of studies has previously reported the therapeutic efficiency of *Aesculus* species against various other cancers (Patlolla *et al.*, 2006; Wang *et al.*, 2010; Yuan *et al.*, 2012; Yuan *et al.*, 2013; Zhou *et al.*, 2009). Here, especially the fractions with strong antioxidant activity more significantly inhibited the growth of cancer cells proposing the anticancer mechanism of the plant. Previously, it has been shown that antioxidants scavenge ROS and free radicals thus not only reducing the growth but also the risk of cancer (Gholamian-Dehkordi *et al.*, 2017; Wu *et al.*, 2017).

In a previously conducted study on *A. turbinata* to determine the antioxidant activity, it was determined that the seed shells were rich in highly polymeric proanthocyanidins compared to the peeled seeds containing high levels of flavanols. From the overall seed extracts, eight flavanol O-glycosides were isolated that exhibited strong antioxidant activity (Kimura *et al.*, 2017). In another study performed on *A. indica* fruit extracts, it was found that the antioxidant activity was due to the presence of phenolic compounds such as quercetin, hydroxyl benzoic acid and mandelic acid (Zahoor *et al.*, 2018). The *A. hippocastanum* flower extracts were studied for their biological activity on vascular endothelial cells and the UPLC-MS analysis determined the presence of high content of polyphenols including flavanoids (Dudek-Makuch *et al.*, 2019). Additionally, the cytotoxic activities from different Aesculus species have been studied with the identification of compounds. The cytotoxic activity from the seeds of *A. chinensis* was studied along with isolation of six new triterpenoid saponins aesculusosides A-F (1-6) which showed potent inhibitory activity against MCF-7 cell line (Cheng *et al.*, 2018). Similarly, thirty-three triterpenoid saponins with fourteen novel compounds aesculiside C-P (1-14) were isolated from the seeds of *A. chinensis* Bge. Var. *chekiangensis* that showed potent cytotoxic activity against HepG2, HCT-116 and MGC-803 tumor cell lines (N. Zhang *et al.*, 2020). The cytotoxic activity of 16 aesculiosides isolated from seeds of *A. glabra* was also investigated against A549 and PC-3 cancer cell lines with GI_{50} from 5.4 to >25µM (Yuan et. al. 2012). Additionally the cytotoxic triterpenoid saponins isolated from *A. californica* showed activity against human non-small cell lung tumor (A549) with GI_{50} ranged from 3.76 to >25µM (Yuan et. al. 2013).

All these studies propose that Aesculus plant family is rich with the presence of active medicinal components

and *A. indica* seeds can be a suitable candidate for bioactive polyphenolic and triterpenoid saponin compounds. These compounds can be further tested against several human carcinoma cell lines.

CONCLUSION

All these findings summarize that the extracts of *A. indica* and its fractions are a rich source of bioactive secondary metabolites that not only play antioxidant role but also inhibit proliferation, migration and colony formation properties of hepatocellular carcinoma cells. However, detailed further studies for isolation of active compounds and their cytotoxicity profile are yet to be done.

REFERENCES

Abdel-Hameed, Bazaid, Shohayeb, El-Sayed and El-Wakil. (2012). Phytochemical studies and evaluation of antioxidant, anticancer and antimicrobial properties of *Conocarpus erectus* L. growing in Taif, Saudi Arabia.*Eur. J. Med. Plants,* pp.93-112.

Aslam and Ahmad (2016). Worldwide importance of medicinal plants: Current and historical perspectives. *Recent. Adv. Biol. Med.,* **2**: 88-93.

Bibi, Nisa, Zia, Waheed, Ahmed and Chaudhary (2012). *In vitro* cytotoxic activity of *Aesculus indica* against breast adenocarcinoma cell line (MCF-7) and phytochemical analysis. *Pak. J. Pharm. Sci.,* **25**(1): 183-187.

Chakraborthy. (2009). Free radical scavenging activity of Aesculus indica leaves. *Int. J. Pharm. Tech. Res.,* **1**(3): 524-526.

Chandra, Sharma and Arora. (2020). Antioxidant compounds from microbial sources: A review. *Food Res. Int.,* **129**:108849.

Cheng Chen, Guo Jiao Cui Wang and bioprospecting. (2018). Triterpenoid saponins from the seeds of *Aesculus chinensis* and their cytotoxicities. *Nat. Prod. Bioprospect.,* **8**(1): 47-56.

Chu Chang, Hsu and Agriculture (2000). Flavonoid content of several vegetables and their antioxidant activity. *J. Sci. Food Agric.,* **80**(5): 561-566.

Da Rocha, Lopes and Schwartsmann. (2001). Natural products in anticancer therapy. *Curr. Opin. Pharmacol.,* **1**(4): 364-369.

Dudek-Makuch, Studzińska-Sroka, Korybalska, Czepulis, Łuczak, Rutkowski and Stobiecki (2019). Biological activity of *Aesculus hippocastanum* flower extracts on vascular endothelial cells cultured *in vitro*. *Phytochem. Lett.,* **30**: 367-375.

Fabricant and Farnsworth. (2001). The value of plants used in traditional medicine for drug discovery. *Environ. Health Perspect.,* **109**(Suppl 1): 69-75.

Farinati, Cardin, Bortolami, Burra, Russo, Rugge and Naccarato (2007). Hepatitis C virus: From oxygen free radicals to hepatocellular carcinoma. *J. Viral Hepatitis,* **14**(12): 821-829.

Firdoos Khan and Ali (2018). Pharmacological Investigation Of Aesculus Indica Aqueous-Ethanol Extract For Its Anti-Nociceptive Action. *J. Anim. Plant Sci.,* **28**(2): 610-615.

Gholamian-Dehkordi, Luther, Asadi-Samani and Mahmoudian-Sani (2017). An overview on natural antioxidants for oxidative stress reduction in cancers; a systematic review. *Immunopathologia Persa,* **3**(2): e12.

Guleria and Vasishth (2009). Ethnobotanical uses of wild medicinal plants by Guddi and Gujjar tribes of Himachal Pradesh. *Ethnobot. Leafl.,* **9**: 8.

Haq Ahmad and Alam (2011). Traditional uses of medicinal plants of Nandiar Khuwarr catchment (District Battagram), Pakistan. *J. Med. Plants Res.,* **5**(1): 39-48.

Hirsch, Iliopoulos, Tsichlis and Struhl (2009). Metformin selectively targets cancer stem cells, and acts together with chemotherapy to block tumor growth and prolong remission. *Cancer Res.,* **69**(19): 7507-7511.

Kanwal Raza, Riaz Manzoor, Tayyeb and Sajid Naseem (2019). Synthesis and characterization of silver nanoparticle-decorated cobalt nanocomposites (Co@ AgNPs) and their density-dependent antibacterial activity. *R. Soc. Open Sci.,* **6**(5): 182135.

Kimura Ogawa, Ishihara Maruoka, Tokuyama-Nakai and Jisaka Yokota (2017). Antioxidant activities and structural characterization of flavonol O-glycosides from seeds of Japanese horse chestnut (*Aesculus turbinata* Blume). *Food Chem.,* **228**: 348-355.

Mandal Ghosh-Choudhury, Yoneda Choudhury and Ghosh-Choudhury (2011). Simvastatin prevents skeletal metastasis of breast cancer by an antagonistic interplay between p53 and CD44. *J. Bio. Chem.,* **286**(13): 11314-11327.

Mishra Sood and Shukla (2018). Phyto-nutritional and mineral composition of Indian Horse Chestnut (*Aesculus indica*) seeds. *J. Pharma. Phytochem.,* **7**(1): 2159-2162.

Navaneetha Krishnan, Rosales Lee and Longevity. (2019). ROS-mediated cancer cell killing through dietary phytochemicals. *Oxid. Med. Cell. Longevity, 2019.*

Newman (2018). From natural products to drugs. *Phys. Sci. Rev.,* **4**(4).

Oroian and Escriche (2015). Antioxidants: Characterization, natural sources, extraction and analysis. *Food Res. Int.,* **74**: 10-36.

Patel Mahobia, Singh Singh, Sheikh Alam and Singh (2010). Antioxidant potential of leaves of *Plectranthus amboinicus* (Lour) Spreng. *Der Pharmacia Lettre,* **2**(4): 240-245.

Patlolla Raju and Swamy Rao (2006). β-Escin inhibits colonic aberrant crypt foci formation in rats and regulates the cell cycle growth by inducing p21waf1/cip1 in colon cancer cells. *Mol. Cancer Ther.,* **5**(6): 1459-1466.

Prasad, Gupta and Tyagi (2017). Reactive oxygen species (ROS) and cancer: Role of antioxidative nutraceuticals. *Cancer Lett.,* **387**: 95-105.

Ramirez-Duron, Ceniceros-Almaguer, Salazar-Aranda, Salazar-Cavazos and De Torres (2007). Evaluation of thin-layer chromatography methods for quality control of commercial products containing *Aesculus hippocastanum, Turnera diffusa, Matricaria recutita, Passiflora incarnata* and *Tilia occidentalis. J. AOAC Int.,* **90**(4): 920-924.

Rates. (2001). Plants as source of drugs. *Toxicon,* **39**(5): 603-613.

Sebolt-Leopold, Dudley, Herrera, Van Becelaere, Wiland, Gowan and Przybranowski (1999). Blockade of the MAP kinase pathway suppresses growth of colon tumors in vivo. *Nat. Med.,* **5**(7): 810-816.

Shah, Shah, Acharya and Acharya (2013). Novel anticancer agents from plant sources. *Chin. J. Nat. Med.,* **11**(1): 16-23.

Sood, Mishra, Sood, & Thakur. (2015). Hypoglycaemic and hypocholesterolimic efficacy of horse chestnut (*Aesculus indica*) using rat model. *J. Clin. Nutr. Diet.,* **1**(1): 1-8.

Wang Ownby, Zhang Yuan Li and letters (2010). Cytotoxicity and inhibition of DNA topoisomerase I of polyhydroxylated triterpenoids and triterpenoid glycosides. *Bioorg. Med. Chem. Lett.,* **20**(9): 2790-2796.

Wu Cheng, Wang and cancer (2017). Dietary antioxidants: potential anticancer agents. *Nutr. Cancer,* **69**(4): 521-533.

Yuan Wang and Deng Li (2012). Cytotoxic triterpenoid saponins from *Aesculus glabra* Willd. *Phytochemistry,* **75**: 67-77.

Yuan Wang and Su Wang Li (2013). Cytotoxic triterpenoid saponins from husks of *Aesculus californica* (Spach) Nutt. *Phytochemistry,* **90**: 95-105.

Zahoor Shafiq and Ullah and Sadiq Ullah (2018). Isolation of quercetin and mandelic acid from *Aesculus indica* fruit and their biological activities. *BMC Biochem.,* **19**(1): 5.

Zhang Li and Lian (2010). An overview of genus Aesculus L.: Ethnobotany, phytochemistry, and pharmacological activities. *Pharmaceutical Crops,* **1**(1): 24-51.

Zhang Wei, Cao Zhang, Kang Ding and Qiu (2020). Bioactive Triterpenoid saponins from the seeds of *Aesculus chinensis* Bge. *var. chekiangensis. Front. Chem.,* **7**: 908.

Zhou Fu, Li Dong, He and Wang (2009). Escin, a natural mixture of triterpene saponins, exhibits antitumor activity against hepatocellular carcinoma. *Planta Med.,* **75**(15): 1580-1585.