ASIAN JOURNAL OF PHARMACEUTICAL AND CLINICAL RESEARCH

INNOVARE ACADEMIC SCIENCES
Knowledge to Innovation

Vol 13, Issue 8, 2020

Online - 2455-3891
Print - 0974-2441

Research Article

LEAVES OF *ANDROGRAPHIS PANICULATA* IS AN ANTIOXIDANT AND ANTICANCER AGENT

S. ANNAI THERASA, G. SOBIYA, S. MABEL PARIMALA*

Department of Zoology, Vector Biology and Pharmacology Research Centre, St. Xavier's College (Autonomous), Palayamkottai, Tamil Nadu, India. Email: mabel_parimala@yahoo.com

Received: 23 April 2020, Revised and Accepted: 06 June 2020

ABSTRACT

Objective: *Andrographis paniculata* (Family: Acanthaceae) is a well-known medicinal plant used in the Indian traditional system of medicine for the treatment of many chronic diseases. The present study was aimed to quantify secondary metabolites, determine antioxidant, and anticancer activity of ethanol extract of *A. paniculata* leaves.

Methods: Leaf sample was macerated with ethanol solvent. Alkaloids, terpenoids, saponins, phenols, and flavonoids were quantified with standard calibrations. The antioxidant potential was tested using 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid (ABTS), and ferric reducing antioxidant power (FRAP) assays. *In vitro* anticancer activity was evaluated using human epithelial type 2 (HEp-2) cell line. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay was used to estimate the cytotoxicity of the extracts. Apoptotic and necrotic effects were characterized by DNA fragmentation assay and fluorescence microscopy using the dual acridine orange/ethidium bromide (AO/EB) staining method.

Results: The phytochemical analysis reveals the presence of alkaloids, saponins, phenols, flavonoids, terpenoids, and steroids. Alkaloids, terpenoids, saponins, phenol, and flavonoid content were recorded as follows: 9.84%, 8.42%, 13.94%, 44.37 mg gallic acid equivalent/100 g, and 904 mg quercetin equivalent/100 g, respectively. The antioxidant activity from DPPH, ABTS, and FRAP assays showed dose-dependent inhibition of free radicals. In cell viability tests, cell death with increasing extract concentration was observed. DNA fragmentation and AO/EB stain confirmed apoptosis and necrosis in extract-treated cells.

Conclusion: The results indicate that *A. paniculata* is a promising source for the development of antioxidant and anticancer drugs.

Keywords: Medicinal plant, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay, DNA fragmentation, Phytochemicals, 2,2-diphenyl-1-picrylhydrazyl, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid, Ferric reducing antioxidant power, Acridine orange/ethidium bromide stain.

 DOI: http://dx.doi.org/10.22159/ajpcr.2020.v13i8.37014

INTRODUCTION

Oxidants and free radicals (reactive oxygen species and reactive nitrogen species) are generated in cells either during normal essential metabolic reactions or derived from external sources. Its overproduction leads to oxidative stress. Increased oxidative stress is widely associated with the development and progression of acute and chronic diseases ranging from infections, diabetes, cancer, and cardiovascular diseases, to other diseases associated with aging [1]. The role of antioxidants is to neutralize the excess of free radicals, to protect the cells against their toxic effects, and to contribute to disease prevention [2]. In the failure of the body's natural antioxidants, the application of synthetic antioxidants to combat oxidative stress has found to be dangerous for human health. Thus, the search for effective, non-toxic natural compounds with antioxidative activity has been intensified in recent years [3]. Phytochemicals from medicinal plants are known to protect the cell against the deleterious effects of free radicals due to their antioxidant activity.

Cancer has become one of the most life-threatening [4] and the leading causes of mortality worldwide in recent years. Among the 200 different cancers that kill humans, laryngeal carcinoma is the cancer of the larynx arising from the squamous cells of the laryngeal epithelium. Smoking is the most contributing factor for laryngeal cancer and death from laryngeal cancer is 20 times more likely for heaviest smokers than for non-smokers [5]. The survival chance of a person becomes better if cancer is detected and treated at earlier stages. Plants have enormous potential to provide newer drugs and as such are a reservoir of natural chemicals that may provide chemoprotective potential against cancer [6]. Phytochemicals play a significant role in impeding the promotion step of multistep carcinogenesis. They also stop or postpone the development of precancerous cells into malignant ones [7].

Andrographis paniculata, (as siriyanangai in Tamil), is one of the most commonly used plants in Unani and Ayurvedic medicine system. It is known as the "king of bitters." It grows in the plains of India and in many other Asian countries. It is also used as a traditional herbal medicine in China, Hong Kong, the Philippines, Malaysia, Indonesia, and Thailand [8]. Conventionally, it is used to overcome different types of fever, difficulty in breathing, homeopathy burning sensation, cough, skin diseases, fever, ulcer, and worms. It is also useful in acidity and liver complaints [9]. Although notable reports against human larynx carcinoma cell lines are available [10,11], no systematic scientific work is reported with *A. paniculata* leaves against HEp-2 cell line until now. Hence, the current study was intended to determine the anticancer activity of the ethanol extract on human larynx carcinoma cell line.

METHODS

Plant collection and extraction

A. paniculata leaves were collected from Ambasamudram, Tirunelveli, in October 2018 and authenticated by Dr. J. John Peter Paul, Assistant Professor, Department of Botany, St. Xavier's College, Tirunelveli. The leaves were cleaned, shade-dried, and powdered with a blender. Ethanol extraction was performed by cold maceration method [12]. In this process, powdered plant material and ethanol in a conical flask were allowed to stand at room temperature for 24 h with frequent agitation. The mixture was strained, the marc pressed, and the liquids

clarified by double-filtration. The obtained *A. paniculata* leaf ethanol (APE) extract was used.

Qualitative phytochemical screening

The presence of phytochemicals was reported following standard procedures [13]. Carbohydrates, proteins and amino acids, phenols, flavonoids, terpenoids, steroids, glycosides, saponins, and alkaloids were detected.

Estimation of secondary metabolites

Total phenolic content

The total phenolic content in APE extract was determined with Folin-Ciocalteau's reagent (FCR) method [14]. In brief, 0.5 ml APE extract was mixed with 2.5 ml FCR (1:10, v/v) followed by 2 ml Na_2CO_3 (7.5%) solution. The tubes were vortexed and allowed to stand for 30 min at room temperature. Absorbance was measured against ethanol blank at 750 nm. Calibrated with gallic acid standard and expressed in grams of gallic acid equivalent per 100 g dry weight.

Total flavonoid content

The total flavonoid content of APE extract was determined by the aluminum chloride colorimetric technique [15]. One milliliter APE extract was mixed with 1 ml distilled water and made up to 10 ml. To this, 0.3 ml $NaNO_2$ (5%) and 0.3 ml $AlCl_3.6H_2O$ (10%) were added. After 6 min incubation at room temperature, 2 ml 1M NaOH was added. The absorbance was measured against ethanol blank at 510 nm, calibrated with quercetin standard, and the results expressed in grams quercetin equivalent per 100 g dry weight.

Saponin content

Saponins were quantified by a standard method with minor modifications [16]. Twenty-gram APE extract was dispersed in 200 ml 20% ethanol. The suspension was heated at 55ºC in a water bath for 4 h. The mixture was filtered and the residue re-extracted with 200 ml 20% ethanol. The combined extracts were reduced to 40 ml at 90°C in a water bath. The concentrate was transferred to 250 ml conical flask and 20 ml ethyl acetate was added and shaken vigorously. The aqueous layer was recovered. Sixty-milliliter n-butanol extracts were washed twice with 10 ml 5% aqueous sodium chloride and dried. The saponin content was expressed in percentage:

$$\text{Total saponin content} = \frac{\text{Weight of saponin obtained}}{\text{Weight of APE extract}} \times 100$$

Alkaloid content

To 5 g of APE extract, 100 ml 10% acetic acid in ethanol was added. The mixture was allowed to stand for 4 h; filtered and concentrated; precipitated with ammonium hydroxide, and the precipitate was collected and washed with ammonium hydroxide [17]. The residue was dried and alkaloid content expressed in percentage:

$$\text{Total alkaloid content} = \frac{\text{Weight of alkaloid obtained}}{\text{Weight of APE extract}} \times 100$$

Terpenoid content

One gram APE extract was taken in a conical flask and soaked in ethyl alcohol for 1 day. Then, it was filtered and extracted with petroleum ether. The ether extract was taken as the measure of total terpenoid [18].

$$\text{Total terpenoid content} = \frac{\text{Weight of terpenoid obtained}}{\text{Weight of APE extract}} \times 100$$

Determination of antioxidant activity

2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay

The antioxidant activity of APE extract was evaluated based on the radical scavenging ability of stable DPPH [19]. DPPH solution (0.004% w/v) was prepared in 95% ethanol and serial dilutions were carried out with the stock solutions (mg/ml) of APE extract. Different concentrations of APE extract were mixed with DPPH solution (2 ml), incubated in the dark for 30 min and absorbance was measured at 517 nm where ethanol (95%) was used as a blank, and ascorbic acid as standard. The percentage of inhibition was calculated:

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

2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid (ABTS) radical scavenging assay

Using ABTS radical, the scavenging ability of APE extract was determined [20]. The stock solution was prepared by mixing 7 mM ABTS and 2.4 mM potassium persulfate and kept in the dark at room temperature for 16 h. The working solution was prepared by diluting with 80% ethanol to obtain an absorbance value 700±0.005. Three hundred microliters of the extract at different concentrations were incubated with 2.7 ml ABTS mixture at 30°C for 30 min and the absorbance was recorded at 734 nm. Ascorbic acid was used as a reference. The scavenging activity of the extract was expressed in percentage:

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

Ferric reducing antioxidant power (FRAP) assay

The FRAP of various concentrations of APE extract was determined, as described by Oyaizu [21]. 2.5 ml 0.2 M phosphate buffer and 2.5 ml 1% $K_3Fe(CN)_6$ are added to 1 ml APE extract dissolved in distilled water. The mixture is incubated at 50°C for 20 min followed by the addition of 2.5 ml 10% trichloroacetic acid. This is centrifuged at 3000 rpm for 10 min to collect 2.5 ml upper layer of the solution, mixed with 2.5 ml distilled water and 0.5 ml 0.1% $FeCl_3$. The absorbance is then measured at 700 nm against a blank sample.

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

Evaluation of anticancer activity

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay

The cytotoxicity of APE extract was tested in the normal Vero cell line and HEp-2 cell line by MTT assay [22]. The cells were seeded in 96-well microplates (1×10^6 cells/well) and incubated at 37°C for 24 h in 5% CO_2 incubator and allowed to grow 80% confluence. Then, the medium was replaced and the cells were treated with APE extract in different concentrations (50, 100, 150, 200, and 250 µg/ml) and incubated for 24 h. The morphological changes of untreated (control) and the treated cells were observed under a bright-field microscope after 24 h and photographed. The cells were then washed with phosphate buffer saline (PBS, pH 7.4) and 20 µl MTT solution (5 mg/ml in PBS) was added to each well. The plates were then allowed to stand at 37°C in the dark for an additional 2–4 h. The formazan crystals were dissolved in 100 µl dimethyl sulfoxide and the absorbance was read at 570 nm. Percentage of cell viability was calculated,

$$\text{Cell viability}(\%) = \frac{\text{Absorbance of sample}}{\text{Absorbance of control}} \times 100$$

DNA fragmentation

The Vero cells and HEp-2 cells were treated with half-maximal inhibitory (IC_{50}) concentration of APE extract for 24 h. The DNA was extracted and the cells were washed with PBS followed by the addition of 0.5 ml lysis buffer and transferred into a microfuge tube. This mixture was incubated for 1 h at 37°C and to this, 4 µl proteinase K was added, incubated at 50°C for 3 h. To each sample, 0.5 ml phenol:chloroform:isoamyl alcohol (25:24:1) was added, mixed, and centrifuged at 10000 rpm for 10 min at 4°C. After centrifugation, to the supernatant, two volumes of ice-cold absolute ethanol and 1/10 volume of 3 M sodium acetate were added and incubated for 30 min on ice to

precipitate DNA. DNA was pelleted by centrifugation at 10,000 rpm for 10 min at 4°C. The supernatant was aspirated and the pellet was washed with 1 ml 70% ethanol. Centrifugation process was repeated until the last traces were removed and the pellet was allowed to dry at room temperature for approximately 30 min and re-suspended in 50 µl of Tris-ethylenediaminetetraacetic acid buffer. DNA samples (10 µg/ml) were run in 1% agarose gel electrophoresis.

Acridine orange (AO)/ethidium bromide (EB) staining method

A fluorescence microscopy study was performed by AO/EB dual staining technique [23]. The Vero cells and HEp-2 cells were seeded in 6-well plates and treated with IC_{50} concentration of APE extract for 24 h. For the nuclear analysis, the monolayer of cells was washed with PBS and stained with 5 µl of AO (100 µg/ml) and 5 µl of EB (100 µg/ml). The morphological changes and nuclei in the stained cells were observed by Floid Cell Imaging fluorescent microscopy.

Analysis

The data of all the tests were analyzed in Microsoft Excel 2010.

RESULTS

Phytochemicals in APE extract

Phytochemicals in APE extract are tabulated (Table 1). Alkaloids, saponins, phenols, flavonoids, and terpenoids were detected.

Phenol, flavonoid, saponin, alkaloid, and terpenoid content in APE extract is presented (Table 2).

Antioxidant activity

In the DPPH assay, APE extract displayed a DPPH radical scavenging effect in a concentration-dependent manner (Fig. 1) with a maximum inhibition of 57.14% at 250 mg/ml APE extract. IC_{50} value was 205.58 mg/ml. However, standard ascorbic acid at 6 mg/ml concentration inhibited 71% radicals (Fig. 2). In ABTS assay, APE extract showed effective ABTS radical scavenging activity in a concentration-dependent manner with inhibition increasing with an increase in concentration from 50 to 250 mg/ml APE extract (Fig. 1). IC_{50} value was 124.35 mg/ml. On the other hand at a concentration as low as 2 mg/ml, ascorbic acid tremendously inhibited (91.89%) ABTS radical (Fig. 2). In FRAP assay, APE extract showed a 98% reduction of oxidized intermediates at 250 mg/ml and recorded less reducing power at lower concentrations (Fig. 1) with an IC_{50} 153.50 mg/ml. Ascorbic acid standard showed a reducing power of 48.02% at the highest concentration (14 mg/ml) tested (Fig. 2).

Table 1: Phytochemicals in APE extract

Phytochemicals	APE extract
Alkaloids	+
Carbohydrates	–
Glycosides	–
Saponins	+
Proteins	–
Amino acids	–
Phenols	+
Flavonoids	+
Terpenoids	+
Steroids	–

"+" indicates presence; "–" indicates absence. APE: *Andrographis paniculata* leaf ethanol

Table 2: Secondary metabolites in APE extract

Chemical constituents	Quantity
Phenol	44.37 mg GAE/100 g
Flavonoid	904 mg QE/100 g
Saponin	13.94%
Alkaloid	9.84%
Terpenoid	8.42%

Phenol content is expressed in gallic acid equivalents; Flavonoid content is expressed in QEs. QE: Quercetin equivalent, GAE: Gallic acid equivalent, APE: *Andrographis paniculata* leaf ethanol

Anticancer activity

Cytotoxicity of APE extract was tested on the HEp-2 human laryngeal cancer cell line and normal Vero cell line using the MTT assay. APE extract is less cytotoxic in the normal Vero cell line. At 250 µg/ml concentration of the extract, 76% of cells were viable (Table 3). While at the same concentration of APE extract in the HEp-2 cell line, only 40% of cells were viable (Table 3), indicating maximum cell death in the cancer cell line.

In DNA fragmentation assay to detect typical ladder or smear appearance, the results show that treatment with APE-extract resulted in the degradation of chromosomal DNA. On running electrophoresis, DNA of Vero cells remain intact as indicated by a single band (Lane 1) while HEp-2 cells show smear (Lane 2). In necrosis, DNA is usually not cut in an orderly manner, which is explained by the activation of non-specific lysosomal nucleases and evidenced by a smear in electrophoresis (Fig. 3).

In AO/EB dual staining technique, the micrograph in green color filter signifies the live-cell present in the HEp-2 cell line and red color filter shows the dead cells present in the HEp-2 cell line (Fig. 4). One of the earliest signs of apoptosis is the loss of plasma membrane integrity. In cells undergoing apoptosis, membrane phospholipid

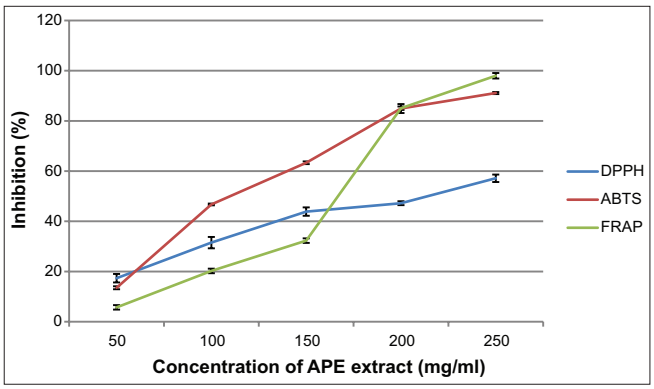

Fig. 1: This line diagram shows the inhibition percentage of *Andrographis paniculata* leaf ethanol extract in five different concentrations using 2,2-diphenyl-1-picrylhydrazyl, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid and ferric reducing antioxidant power assays. Values are plotted in mean±SD of triplicates

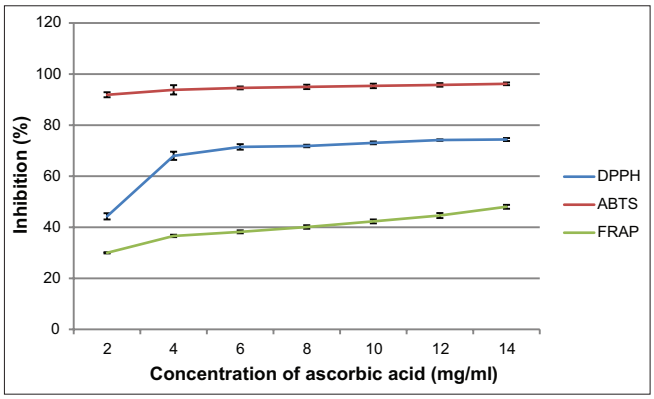

Fig. 2: This line diagram shows the inhibition percentage of standard ascorbic acid in seven different concentrations using 2,2-diphenyl-1-picrylhydrazyl, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid, and ferric reducing antioxidant power assays. Values are plotted in mean±SD of triplicates

Table 3: Cell viability with *Andrographis paniculata* leaf ethanol extract

Sample (µg/ml)	Vero	Hep-2
Control	100	100
50	93.57±0.82	93.78±0.05
100	91.57±0.74	81.80±0.16
150	87.68±0.04	63.49±1.00
200	83.51±0.64	48.95±0.57
250	75.78±0.23	39.94±0.61

Values are expressed in mean±SD of triplicates

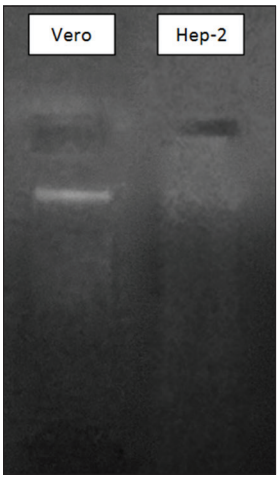

Fig. 3: This image shows the agarose gel electrophoresis of crude DNA. Lane 1 is the DNA extracted from Vero cell line displaying a single band; Lane 2 is the DNA extracted from HEp-2 cell line forming a smear

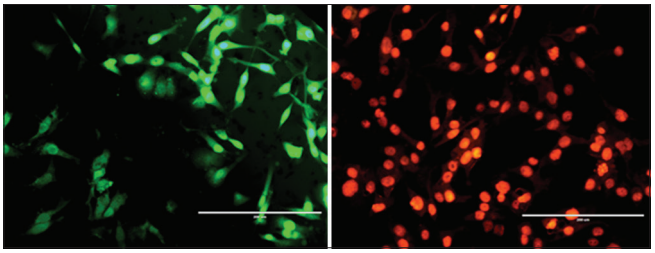

Fig. 4: This micrograph shows HEp-2 cells as observed by dual acridine orange/ethidium bromide staining technique. Green filter signifies cells undergoing apoptosis; red filter exhibits dead cells

phosphatidylserine is translocated from the inner surface to the outer surface of the plasma membrane. The photograph displays a moderate amount of apoptosis because of APE extract.

DISCUSSION

Oxidative stress is the main causative factor for diabetes, rheumatoid arthritis, cardiovascular diseases, atherosclerosis, neurodegenerative diseases, cancer, and aging. Natural antioxidants have been reported from plants that offer resistance against oxidative stress by scavenging free radicals, inhibiting lipid peroxidation, and by other mechanisms [24]. Thus, the present study was sought to register the antioxidant and anticancer property of *A. paniculata* ethanol leaf extract.

In the present study, preliminary phytochemical analysis of APE extract indicates the presence of alkaloids, saponins, phenols, flavonoids, and terpenoids. *A. paniculata* is reported to contain diterpenes, lactones, and flavonoids. Constituents observed in our study are as per the previous study in aqueous, methanol, petroleum ether, and chloroform extracts of *A. paniculata* [25]. We have quantified saponins, alkaloids, terpenoids, phenols, and flavonoids. In an earlier study, quantitative phytochemical analysis with two extracts (aqueous and ethanol) of *A. paniculata,* the mean concentration of tannins, terpenoids, and saponins was higher in aqueous extract whereas alkaloids, total phenols, non-tannin phenolic and flavonoids were higher in the ethanolic extract [26].

Since plant antioxidants are often considered to protect against different ailments [27,28], the antioxidant property of APE extract was tested with three widely known methods (DPPH, ABTS, and FRAP). Indeed each method involves a different mechanism. Irrespective of the methods used, APE extract had antioxidant activity. In the DPPH assay, an odd electron displays a strong absorption band at a wavelength of 519 nm, which loses absorption once the odd electron is paired off by hydrogen or electron-donating antioxidant [29]. Our DPPH activity is concurrent with another study with *A. paniculata* leaves hexane, ethyl acetate, chloroform, and methanol extracts in concentrations ranging from 25 to 100 mg/ml which has reported an increase in inhibition with increasing concentration [30]. In ABTS, the reduction of the blue-green ABTS radical by hydrogen-donating antioxidants is measured by the suppression of its characteristic long-wave absorption spectrum [31]. The % inhibition of ABTS by the ethanol leaf extract of *A. paniculata* was concentration-dependent and similar results were observed in a study that compared activity with the rutin and Vitamin E [32]. The FRAP mechanism is based on the ability of antioxidants to reduce Fe^{3+} to Fe^{2+} in a low pH medium that decreases ionizing potential which drives hydrogen atom transfer and increases the redox potential which is the dominant reaction mechanism [33]. Our FRAP activity is in agreement with a previous study in *A. paniculata* leaves methanol extract with an IC_{50} of 210 µg/ml [34]. Ascorbic acid used as a standard in most of the antioxidant studies [35] was used in our study.

The anticancer activity was tested with MTT assay. This assay is extensively used to measure cell proliferation, test cytotoxic effects of compounds, and to multiplex as an internal control to determine viable cell number during other cell-based assays [36]. Our results indicate that APE extract was more toxic to the HEp-2 cell line than to normal Vero cells. A similar MTT study in different concentrations was reported on the MCF-cell line with nanoparticles [37].

When cytotoxicity was confirmed, we wanted to test the mechanism by which APE extract killed the cells. Of the three main means by which cells die, the genetically regulated apoptotic pathway [38] is much known. Autophagy, or digestion of cell on its own, is also recognized as a form of regulated cell death [39]. The third type, necrosis, is an unregulated and accidental form of cellular death. There is increasing evidence that cells also use necrosis in a programmed genetic fashion to die [40]. DNA ladder assay detects apoptosis [41]. DNA fragmentation is a hallmark of apoptosis that distinguishes apoptotic death from necrotic death. Treatment of cells with APE extract caused DNA smear in the second lane of electrophoresis gel, suggesting that the cell has undergone necrosis. Cancer cells can die from necrosis in response to the chemotherapy, and activate the innate immune response and possibly, if there are cancer-specific antigens, activate a response against the remaining cancer cells, providing a potential way for the immune system to actively deal with the remaining cancer cells [42]. HEp-2 cell was also tested by AO/EB dual staining technique. Under the fluorescent microscope, live cells appear uniformly green and apoptotic cells stain green but contain bright green dots in the nuclei as a consequence of chromatin condensation and nuclear fragmentation; necrotic cells stain orange, but the fluorescent is weak or even disappear [23]. In a very recent study, andrographolide, a natural diterpenoid from *A. paniculata* along with carboplatin, is reported to synergistically inhibit cell proliferation and induce mitochondrial apoptosis of Hep-2 cells by increasing the intracellular reactive oxygen species, regulating the mitogen-activated protein kinase and phosphatidylinositol 3-kinase (PI3K/AKT) pathways, and ultimately activating the cleavage of Caspase-3 [43]. Interestingly, APE extract-treated cells in our study showed both apoptosis and necrosis. Thus, in our study, the crude *A. paniculata* ethanol leaf extract has activated both apoptosis and necrosis pathway to kill the cancerous cells.

CONCLUSION

It can be concluded that *A. paniculata* can be a source for the isolation, identification, and development of novel and effective anticancer and antioxidant drugs. However, further studies are needed to substantiate the *in vivo* potential of this plant in the management of oxidative stress and cancer.

AUTHORS' CONTRIBUTIONS

S. Annai Therasa: Performed quantification and antioxidant assays. G. Sobiya: Carried out anticancer assays. S. Mabel Parimala: Conceptualization of the work, interpretation of results, and preparation of manuscript.

CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

AUTHORS' FUNDING

The authors acknowledge that the present study was not funded by any research institute or organization and the expense was met by the authors concerned.

REFERENCES

1. Uttara B, Singh AV, Zamboni P, Mahajan RT. Oxidative stress and neurodegenerative diseases: A review of upstream and downstream antioxidant therapeutic options. Curr Neuropharmacol 2009;7:65-74.
2. Pham-Huy LA, He H, Pham-Huy C. Free radicals, antioxidants in disease and health. Int J Biomed Sci 2008;4:89-96.
3. Lobo V, Patil A, Phatak A, Chandra N. Free radicals, antioxidants and functional foods: Impact on human health. Pharmacogn Rev 2010;4:118-26.
4. Tagne RS, Telefo BP, Nyemb JN, Yemele DM, Njina SN, Goka SM, *et al*. Anticancer and antioxidant activities of methanol extracts and fractions of some Cameroonian medicinal plants. Asian Pac J Trop Med 2014;7S1:S442-7.
5. Kumar A, Selvakumar S. Antiproliferative efficacy of *Tabernaemontana divaricata* against HEp-2 cell line and Vero cell line. Pharmacogn Mag 2015;11 Suppl 1:S46-52.
6. Desai AG, Qazi GN, Ganju RK, El-Tamer M, Singh J, Saxena AK, *et al*. Medicinal plants and cancer chemoprevention. Curr Drug Metab 2008;9:581-91.
7. Meybodi NM, Mortazavian AM, Monfared AB, Sohrabvandi S, Meybodi FA. Phytochemicals in cancer prevention: A review of the evidence. Int J Cancer Manag 2017;10:e7219.
8. Akbar S. *Andrographis paniculata*: A review of pharmacological activities and clinical effects. Altern Med Rev 2011;16:66-77.
9. Rajalakshmi V, Cathrine L. Phytochemical screening and antimicrobial activity of ethanolic extract of *Andrographis paniculata*. J Pharmacogn Phytochem 2016;5:175-7.
10. Patel PR, Nagar AA, Patel RC, Rathod DK, Patel VR. *In vitro* anticancer activity of *Rubia cordifolia* against HeLa and HEp-2 cell lines. Int J Pharm Pharm Sci 2011;3 Suppl 2:70-1.
11. Ramesh S, Dilipan E, Mayavu P. Effects of drugs against antioxidant and cytotoxic (HEp-2 cell line) activity compound from marine animals *Conus amadis* venom (Gmelin, J.F, 1791). Int J Pharm Pharm Sci 2014;6:638-43.
12. Handa SS. An overview of extraction techniques for medicinal and aromatic plants. In: Handa SS, Khanuja SP, Longo G, Rakesh DD, editors. Extraction Technologies for Medicinal and Aromatic Plants. Trieste: ICS-UNIDO; 2008. p. 21-54.
13. Trease GE, Evans WC. Pharmacognosy. 12th ed. London: Balliere Tindall; 1983.
14. Demiray S, Pintado ME, Castro PM. Evaluation of phenolic profiles and antioxidant activities of Turkish medicinal plants: *Tilia argentea*, *Crataegi folium* leaves and *Polygonum bistorta* roots. World Acad Sci Eng Technol 2009;54:312-7.
15. Chang CC, Yang MH, Wen HM, Chern JC. Estimation of total flavonoid content in *Propolis* by two complementary colorimetric methods. J Food Drug Anal 2002;10:178-82.
16. Obadoni BO, Ochuko PO. Phytochemical studies and comparative efficacy of the crude extracts of some haemostatic plants in Edo and Delta states of Nigeria. Glob J Pure Appl Sci 2002;8:203-8.
17. Harborne JB. Phytochemical Methods: A Guide to Modern Techniques of Plant Analysis. London: Chapman and Hall; 1973.
18. Ferguson NM. A Textbook of Pharmacognosy. New York: The Macmillon Company; 1956. p. 191.
19. Braca A, Sortino C, Politi M, Morelli I, Mendez J. Antioxidant activity of flavonoids from *Licania licaniaeflora*. J Ethnopharmacol 2002;79:379-81.
20. Arnao M, Cano A, Acosta M. The hydrophilic and lipophilic contribution to total antioxidant activity. Food Chem 2001;73:239-44.
21. Oyaizu M. Studies on products of browning reaction: Antioxidative activity of products of browning reaction prepared from glucosamine. Jpn J Nutr Diet 1986;44:307-15.
22. Mosmann T. Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. J Immunol Methods 1983;65:55-63.
23. Liu K, Liu PC, Liu R, Wu X. Dual AO/EB staining to detect apoptosis in osteosarcoma cells compared with flow cytometry. Med Sci Monit Basic Res 2015;21:15-20.
24. El-Jemli M, Kamal R, Marmouzi I, Zerrouki A, Cherrah Y, Alaoui K. Radical-scavenging activity and ferric reducing ability of *Juniperus thurifera* (L.), *J. oxycedrus* (L.), *J. phoenicea* (L.) and *Tetraclinis articulata* (L.). Adv Pharmacol Sci 2016;2016:6392656.
25. Das P, Srivastav AK. Phytochemical extraction and characterization of the leaves of *Andrographis paniculata* for its anti-bacterial, anti-oxidant, anti-pyretic and anti-diabetic activity. Int J Innov Res Sci Eng Technol 2014;3:15176-84.
26. Nagajothi S, Mekala P, Raja A, Raja MJ, Senthilkumar P. *Andrographis paniculata*: Qualitative and quantitative phytochemical analysis. J Pharmacogn Phytochem 2018;7:1251-3.
27. Chintalapani S, Swathi MS, Narasu ML. Phytochemical screening and *in vitro* antioxidant activity of whole plant extracts of *Sesuvium portulacastrum* L. Asian J Pharm Clin Res 2018;11:322-7.
28. Jassal PS, Sharma M. Evaluation of antioxidant, antibacterial, antihemolytic, and phytochemical properties of *Ficus benjamina*, *Ficus infectoria* and *Ficus krishnae*. Asian J Pharm Clin Res 2019;12:68-73.
29. Liang N, Kitts D. Antioxidant property of coffee components: Assessment of methods that define mechanisms of action. Molecules 2014;19:19180-208.
30. Sangeetha S, Archit R, Mythili S, Sathiavelu A. A detailed analysis of the antioxidant activity of the medicinal plant *Andrographis paniculata*. Int J Drug Dev Res 2014;6:231-8.
31. Ratnavathi CV, Komala VV. Sorghum grain quality. In: Ratnavathi CV, Patil JV, Chavan UD, editors. Sorghum Biochemistry: An Industrial Perspective. London: Elsevier; 2016. p. 1-61.
32. Adeoye BO, Akinrinde AS, Oyagbemi AA, Omobowale TO, Afolayan AJ, Adedapo AA. Phytochemical profiling, antioxidant activities and essential oil constituents of *Andrographis paniculata*. Trop Vet 2018;36:52-64.
33. Cerretani L, Bendini A. Rapid assays to evaluate the antioxidant capacity of phenols in virgin olive oil. In: Preedy VR, Watson RR, editors. Olives and Olive Oil in Health and Disease Prevention. London: Elsevier; 2010. p. 625-35.
34. Prakash SE, Ali SH, Divya N, Rani RV, Manavalan R. Evaluation of *in vitro* antioxidant activity of leaf extract of *Andrographis paniculata*. Res J Pharm Biol Chem Sci 2011;2:891-5.
35. Adedapo AA, Adeoye BO, Sofidiya MO, Oyagbemi AA. Antioxidant, antinociceptive and anti-inflammatory properties of the aqueous and ethanolic leaf extracts of *Andrographis paniculata* in some laboratory animals. J Basic Clin Physiol Pharmacol 2015;26:327-34.
36. Riss TL, Moravec RA, Niles AL, Duellman S, Benink HA, Worzella TJ, *et al*. Cell viability assays. In: Sittampalam GS, Grossman A, Brimacombe K, Arkin M, Auld D, Austin CP, *et al*, editors. Assay Guidance Manual. Bethesda, MD: Eli Lilly and Company and the National Center for Advancing Translational Sciences; 2004.
37. Amudha CK, Deeba F, Rajarajan P. Characterization and anti-neoplastic potential of phytofabricated silver nanoparticles on human breast cancer cell line (MCF-7). Int J Appl Pharm 2019;11:70-7.
38. Danial NN, Korsmeyer SJ. Cell death: Critical control points. Cell 2004;116:205-19.
39. Gozuacik D, Kimchi A. Autophagy as a cell death and tumor suppressor mechanism. Oncogene 2004;23:2891-906.
40. Proskuryakov SY, Konoplyannikov AG, Gabai VL. Necrosis: A specific form of programmed cell death? Exp Cell Res 2003;283:1-16.
41. Saadat YR, Saeidi N, Vahed SZ, Barzegari A, Barar J. An update to DNA ladder assay for apoptosis detection. Bioimpacts 2015;5:25-8.
42. Goodman L. The kiss of death. J Clin Invest 2004;113:1662.
43. Mao W, He P, Wang W, Wu X, Wei C. Andrographolide sensitizes HEp-2 human laryngeal cancer cells to carboplatin-induced apoptosis by increasing reactive oxygen species levels. Anticancer Drug 2019;30:e0774.