**Tamarix articulata** Inhibits Cell Proliferation, Promotes Cell Death Mechanisms and Triggers G₀/G₁ Cell Cycle Arrest in Hepatocellular Carcinoma Cells

Running head: Evaluation of Anticancer Potential of *Tamarix articulata*

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**SUMMARY**

*Research background.* From ancient times plants have been used for medicinal purposes against various ailments. In the modern era, plants are a major source of drugs and are an appealing drug candidate for the anticancer therapeutics against various molecular targets. Here we tested *Tamarix articulata* methanolic extract of dry leaves for anticancer activity against a panel of hepatocellular carcinoma cells.

*Experimental approach.* Cell viability of hepatocellular carcinoma cells was determined by MTT assay after dose-dependent treatment with extract of *Tamarix articulata*. Phase-contrast microscopy and DAPI staining were performed to analyze cellular and nuclear morphology. Immunoblotting was performed to determine the expression of proteins associated with autophagy, apoptosis, and cell cycle. However, flow cytometry was used for the quantification of apoptotic cells and the analysis of cells in different phases of the cell cycle after treatment with varying doses of *Tamarix articulata*. Additionally, acridine orange staining and DCFHDA dye were used to analyze the quantification of autophagosomes and reactive oxygen species.

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Results and conclusion. Our results demonstrate that *Tamarix articulata* methanolic extract exhibits promising antiproliferative activity with IC$_{50}$ values (271±4.38), (298±7.08) and (336±6.11) µg/mL against HepG2, Huh7D12, and Hep3B cell lines, respectively. Mechanistically, we found *Tamarix articulata* methanolic extract induces cell death by activating apoptosis and autophagy pathways. First, *Tamarix articulata* methanolic extract promotes autophagy which was confirmed by acridine orange staining. The immunoblotting analysis further confirms that *Tamarix articulata* methanolic extract consistently induces the conversion of LC3I to LC3II form with a gradual decrease in expression of autophagy substrate protein p62 at higher doses. Second, *Tamarix articulata* methanolic extract promotes reactive oxygen species production in hepatocellular carcinoma cells and activates reactive oxygen species-mediated apoptosis. Flow cytometry and immunoblotting analysis showed that *Tamarix articulata* methanolic extract induces dose-dependent apoptosis and activates proapoptotic proteins caspase-3 and PARP1. Additionally, *Tamarix articulata* methanolic extract triggers the arrest of the G0/G1 phase of the cell cycle and upregulates the protein expression of p27/Kip, and p21/Cip, with a decrease in cyclin-D1 expression in hepatocellular carcinoma cells.

Novelty and scientific contribution. The current study demonstrates that *Tamarix articulata* methanolic extract exhibits promising anticancer potential to kill tumor cells by programmed-cell-death type I and II mechanisms and could be explored for potential drug candidate molecules to curtail cancer in the future.

Key words: *Tamarix articulata*, autophagy, apoptosis, cell cycle, antiproliferative activity

INTRODUCTION

Hepatocellular carcinoma (HCC) is the predominant primary cancer in most countries, and seventh-most frequent cancer across the globe (1). HCC has the second most lethal cancer-associated mortality in the world, with an annual rate of around 1.2 million deaths, mainly in under-developing and developing countries of East Asia and Africa (2). The incidence of HCC varies according to gender, age, ethnicity, and geographical distribution. The majority (more than 80%) of HCC cases occur in African and East Asian countries with an incidence rate of more than 20 people in 100,000 individuals. HCC is rarely appearing below the age of 40 and males are at high risk than females (3). Several etiological factors that have a casual association with HCC are chronic viral infections such as hepatitis B virus (HBV) or hepatitis C virus (HCV), frequent exposure to aflatoxin B1, liver cirrhosis due to alcohol abuse, and non-alcoholic fatty liver (4). Patients with early disease are often asymptomatic and consequently diagnosed at a late stage when the disease is untreatable (5).
At the molecular level, HCC is a complex and heterogeneous due to several key mutations. These mutations cause aberrant modulations of key proteins that result in the deregulation of crucial signaling pathways (6,7). Owing to many genetic mutations in HCC and the deregulation of many signaling pathways, HCC is often resistant to the current regiment of therapeutic modalities (8). The therapeutic options are surgical resection and are often more successful if the malignancy is diagnosed at an early stage of the disease (9). Although liver transplantation is a best effective therapeutic approach to deal with HCC, however, around 5 % of patients can be benefited from this approach because of histocompatibility and organ availability issues (10). The other therapeutic options are chemotherapy and radiotherapy. Regrettably, HCC resists most cytotoxic drugs. Among chemotherapeutic drugs, sorafenib, a kinase inhibitor is the drug of choice against HCC and increases patient survival time for only a few months (11).

From the last two decades, various novel synthetic chemotherapeutic drugs are used in clinical settings to treat cancer but still not much success has been achieved due to considerable cost of development and off-target deleterious effects on normal cells which causes serious adverse effects like gastrointestinal upset, suppression of immune system, and the development of drug resistance thereby leads to failure of these drugs (12). Thus, identification and evaluation of plant-based novel extracts or compounds are seemingly important.

From the past four decades, natural products obtained from the plant kingdom have received great attention for promising chemotherapeutic potential and are the prime source of medicines for other ailments. Owing to their structural complexity, natural products can hit multiple targets at various stages of tumorigenesis and associated inflammation (13). This provides evidence that natural products are promising anticancer molecules to target deregulated signaling pathways at multiple sites to curtail cancer. The major source of these natural products is derived from plants. More than 60 % of anticancer drugs to treat cancer are derived from plant-based natural products or their derivatives (14). At present around 3000 plants across the globe have been reported to have anticancer potential. Globally, around 10 % to 40 % of plant-derived products are used for the treatment of cancer and is expected to reach 50 % in Asia (15). Therefore, there is a continuous demand to search and development of novel and effective drugs from natural sources (plant sources) which specifically kills tumor cells with less undesirable effects against normal cells.

Intending to search novel compounds with less deleterious effects, we emphasized on natural medicines. Owing to have great medicinal value of plants and plant-derived products for the anticancer therapeutics, one such plant in the deserts of Saudi Arabia containing a variety of secondary metabolites is *Tamarix articulata* (TA) (16). The plant belongs to the family *Tamaricaceae* and is a halophytic plant growing in extremely harsh and arid conditions in the deserts of Saudi Arabia
Traditionally, the plant is used to treat various ailments such as gastrointestinal diseases, skin diseases, heart diseases, and other ailments (17). The major chemical constituents of TA extract of dry leaves are quinic acid, gallic acid, kaempferol, quercetin, Tamarixetin, epicatechin gallate and epiafzelechin. These phytochemicals are responsible for pharmacological properties including antioxidant, antibacterial, anticancer, antidiabetics and hepatoprotective activity (16). Crude extracts of the plant have been reported to exhibit anticancer activities by inhibiting cell viability in various types of cancer cells in other parts of the world (18). Although, our recent study reveals that methanolic extracts of all parts (root, stem, dry and fresh leaves) of TA collected from the Qassim region of Saudi Arabia have promising antioxidant activity due to the presence of polyphenolic and flavonoid compounds. Additionally, we document that all methanolic extracts of different parts of TA exhibit potential anticancer effect, however, the data is preliminary and without any mechanistic study. Therefore, in the current study, we evaluated the antiproliferative activity of TA methanolic extract of dry leaves against a panel of HCC cells (HepG2, Hep3B, and Huh7D12) and to better understand the underlying anticancer mechanism. We observe TA methanolic extract simultaneously induces apoptosis and autophagy in HCC (Hep2G) cells. Additionally, TA methanolic extract triggers the arrest of the G0/G1 phase of the cell cycle and increases the protein expression of Cyclin-dependent kinase inhibitor 1B (p27/Kip), encoded by the CDKN1B gene and cyclin-dependent kinase inhibitor 1 (p21/Cip), encoded by the CDKN1A gene, with a decrease in the expression of cyclin D1 in Hep2G cells. Together these results proved that the medicinal plant TA can be explored for potential drug candidate molecules to curtail cancer in near future.

MATERIALS AND METHODS

Collection of plant material and extract preparation

The plant material (TA) was collected in August 2019 from desert regions of Qassim province of the Kingdom of Saudi Arabia. The plant material (dry leaves) was airdried under the shade to remove moisture. The methanolic extract of dry leaves was prepared as per the standard protocol mentioned in our previous publication (19).

Cell culture and treatments

HCC cells (HepG2, Hep3B, and Huh7D12) and normal transformed cells THLE-2 were ordered and procured from the American Type Culture Collection (ATCC) (Manassas, Virginia, United States). All the procured cell lines were cultured in the required medium (minimum essential medium-MEM; Thermo Fisher Scientific, Waltham, Massachusetts, United States) and maintained
under sterile conditions of temperature (37 °C) in a 5 % CO₂ incubator (New Brunswick Scientific, United Kingdom). The growth medium is added with 1 % penicillin-streptomycin (pen-strep) (Thermo Fisher Scientific, Waltham, Massachusetts, United States) and 10 % fetal bovine serum (FBS) from Gibco (Charlemont Terrace, Crofton Rd, Dún Laoghaire, Dublin, Ireland) to supply essential growth factors for the proper growth of cells. The procured cells were periodically evaluated for Mycoplasma contamination.

**Preparation of TA stock solution**

The stock solution 10 mg/mL of TA methanolic extract of dry leaves was made in Dimethyl sulfoxide and aliquoted, stored at -20 °C in sterile micro-centrifuge (1.5 mL) tubes. At the time of experiment stock concentration was diluted serially in culture media to attain the working concentration in a range of 10 µg/mL and 10000 µg/mL for the treatment of HCC (HepG2, Hep3B, and Huh7D12) cells.

**Chemicals and antibodies**

Propidium iodide (PI), rapamycin, camptothecin, 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT), hydrogen peroxide (H₂O₂), 4',6-diamidino-2-phenylindole (DAPI), staurosporine, 7'-dichlorofluorescein diacetate (DCFHDA), dimethyl sulphoxide (DMSO), and N-acetylcysteine (NAC) procured from Sigma Aldrich (St. Louis, Missouri, United States). The required primary antibodies (cyclin D1, β-actin, p27, p21, MAPLC3-I/II, SQSTM1/P62, PARP1, caspase-3) were procured from Cell Signaling Technology (Dellaertweg 9b, 2316 WZ Leiden, Netherland). The enzyme-labelled mouse and rabbit IgG secondary antibodies were procured from Santa Cruz Biotechnology (Dallas, Texas, United States).

**Cell proliferation/viability assay**

The cell viability was determined by the most commonly used MTT assay as per the standard protocol (20). After trypsinization of HCC cells (HepG2, Hep3B, Huh7, and THLE-2) from the primary cell culture flask, the cells were uniformly suspended in culture media and distributed in 96-well plate at a cell density of 2·10³ cells per well. After overnight incubation, the cells were exposed to varying doses of TA methanolic extract along with DMSO control for 24 h. The cells plated in 96-well plates and were flooded with 20 µl of 2.5 mg/mL of MTT dye solution at 37 °C for 3-4 h. The MTT dye interacts with succinyl dehydrogenase of live mitochondria to form formazan crystals. The purple color formation developed after dissolving the formazan crystals in DMSO. Subsequently, the absorbance of the coloured solution was measured at 570 nm with the help of the multi-plate reader. The
percentage of viable cells was analyzed by GraphPad Prism 5.0 software (21) to calculate the percentage of cell proliferation of the treated group compared to the DMSO control group.

**Apoptosis detection using flow cytometry**

Annexin V-FITC Apoptotic detection kit was employed to analyze apoptosis using flow cytometry (22). In Brief, HCC (HepG2) cells 5·10⁵ cells were harvested and plated in each well of the 6-well plate and allow them to adhere overnight to the surface properly. The cells were exposed to varying doses of TA methanolic extract in the presence of untreated control for 24 h under sterile conditions of temperature 37 °C and CO₂ (5 %). Next, cells were harvested, washed with ice-cold PBS 3 times, and resuspend the cells in binding buffer solution followed by the addition of 5 µl each of FITC-conjugated annexin V and PI for 10-15 min at 4 °C in dark. After, incubation samples were analyzed using flow cytometry (BD FACS Calibur; BD Bioscience) to quantify the apoptotic cell population.

**Cell cycle analysis**

Briefly, 5·10⁵ HepG2 cells were harvested and plated in each well of the 6-well plate and allow them to adhere properly (23). The cells were exposed to varying concentrations (125, 250, 500 mg/mL) of TA methanolic extract along with untreated control at 37 °C in a 5 % CO₂ incubator for 24 h. The treated cells in the 6-well plate were harvested, washed, and were fixed in 70 % ice-cold ethanol overnight at 4 °C. Next day the cells were processed for cell cycle analysis by incubating fixed cells with RNase (100 µg/mL) for 30 minutes at 37 °C and stained with PI (50 µg/mL) in the dark for another 30 min at 4 °C. The cell cycle analysis was done by using the BDTM LSRII flow cytometry system (BD Bioscience, Franklin Lakes, USA) to analyze cells in different phases of the cell cycle.

**Phase-contrast microscopy**

HCC (HepG2) cells (5·10⁵) were seeded on coverslips in 30 mm Petri-dishes and were exposed to different concentrations of TA methanolic extract of for 24 h (24). After completion of the treatment period, cells were processed for phase-contrast microscope LSM-510 (Carl Zeiss, Germany) to detect the phenotypic changes in the morphology of treated cells.

**Immunoblotting**

Briefly, 0.5·10⁶ HepG2 cells were harvested and seeded in each well of the 6-well plate overnight to get adhered properly as per the standard protocol (25). The plated HCC (HepG2) cells were treated with different doses of TA methanolic extract (125, 250, 500 mg/mL) along with control
DMSO for 24 h. After treatment, the cells were harvested, washed with PBS, and were resuspended in cell lysis buffer. Resuspended cells in the cell lysis buffer were constantly vortexed for 10 seconds and kept in ice for at least 2 min and repeat the step for at least five times. The cell lysis solution obtained was subjected to centrifugation (12,000 g, 5810 R, Eppendorf, Chennai, India) at 4 °C for 10 min. The supernatant obtained is collected in separate autoclaved microtubes, followed by protein estimation by standard Bradford method. An equal amount of protein sample (30 µg) obtained from various treatments were loaded in each well of pre-casted SDS-PAGE gel and allow the gel to resolve based on molecular weight at a standard voltage and current. Transfer the properly resolved proteins of SDS-PAGE gel on to the PVDF membrane. The PVDF membrane is incubated with a 5 % blocking solution containing fat-free milk in TBST to block non-specific antibody binding sites. The PVDF membrane was incubated with the primary antibody at the desired dilution at room temperature for 3-4 h or overnight at 4 °C to ensure proper binding with respective protein/antigen. After completion of incubation, the PVDF membrane is subjected to gentle washing with TBST buffer for at least five times and was again probed with enzyme-tagged IgG secondary antibody for 1 h at 37 °C. The membrane was again gently washed with TBST for at least five times. The membrane was flooded and incubated in enhanced chemiluminescence plus substance to spot the presence of protein with the help of x-ray film or with chemiluminescence inbuilt gel doc.

Reactive oxygen species (ROS) determination

ROS determination was performed by commonly used DCFHDA dye into the green, fluorescent dichlorofluorescein (DCF) as per the standard protocol (26). Briefly, 1.0·10^5 HCC (HepG2) cells were harvested after trypsinization and plated in each well of a 6-well plate overnight to get attached to the surface properly. The cells plated in wells were exposed to varying doses of TA methanolic extract in the presence and absence of NAC (5 mM) along with untreated control and positive control H_2O_2 at 37 °C for 24 h. The treated cells in each well were gently washed with ice-cold PBS and incubated with DCFHDA dye for 30 min at 37 °C in a 5 % CO_2 incubator for staining. Subsequently, stained cells were photographed under a fluorescent microscope and plate were analyzed by a fluorescence microplate reader at 488 nm excitation wavelength and 525 nm emission wavelength.

Autophagosome staining.

The detection of autophagosomes by microscopy was determined by standard protocol (27). Briefly, 1.0·10^5 HCC (HepG2) cells were harvested after trypsinization and plated in each well of a 6-well plate overnight to get attached to the surface properly. The cells plated in wells were exposed to
varying doses (125, 250, 500 µg/mL) of TA methanolic extract along with untreated control and positive control rapamycin (100 nM) at 37 °C for 24 h. Next, the cells containing TA methanolic extract in culture medium were removed and fresh medium containing acridine orange (AO) (1 mg/mL) were added and incubated for 15 min at 37 °C in 5 % CO₂. The AO-stained cells in each well of 6-well were analyzed under a fluorescent microscope, due to low or acidic pH in autophagosomes appears bright red and count the cells per field from many fields to a minimum of 100 cells to determine the percentage of cells promotes autophagosome formation.

**Nuclear morphology by DAPI staining**

Detection of nuclear morphology was analyzed by DAPI staining (28). Briefly, 50·10^3 HCC (HepG2) cells were seeded and plated in the chamber well slide and let them adhere properly to the surface of the chamber slide. Next, morning HCC (HepG2) cells were treated with different doses of TA methanolic extract along with untreated control and positive control (camptothecin 2 µM) for 24 h. Cells were rinsed with pre-cold PBS, followed by fixing and permeabilization with 4 % and triton X-100, respectively. Finally, fixed cells were mounted with ultracruz mounting media and were analyzed by a microscope for the detection of nuclear morphology.

**Statistical analysis**

All the experimentations were executed more than three times. The statistical analysis of all experiments was performed by using GraphPad Prism software (21) from three independent experiments. All the results obtained represent the mean of ±SEM, calculated and processed by one-way ANOVA. The *p*-value equal to or less than 0.05 were designated as significant.

**RESULTS AND DISCUSSION**

Plant-based extracts or natural compounds derived from plant extracts with chemopreventive activity are a vital source of drugs against many diseases including cancer (29). Approximately more than 50 % of the current regimen of the Food and Drug Administration (FDA) approved drugs are derived from plants (30). Yet, there are numerous plants of various species that could have the potential to exhibit pharmacological activities and can be the important source of novel compounds for future therapeutics with great efficacy against tumor cells and fewer toxicity issues against normal human cells. Plants contains numerous polyphenolic compounds which exhibits pharmacological properties. TA is wild plant growing in the deserts of Saudi Arabia and contains a variety of secondary metabolites. Belongs to *Tamaricaceae* family, TA is a halophytic plant growing in the extremely harsh environmental conditions in the deserts of Saudi Arabia (16). Since ancient times TA has been utilized
as a traditional medicine to cure several inflammatory ailments which include gastrointestinal disorders, ulcerations, skin diseases, and hair loss (16). However, in the recent past, TA has been reported to exhibit some promising biological activities such as antioxidant, antidiabetic, hypolipidemic, antibacterial, and hepatoprotective activity (18,19,30–32). A plethora of findings exhibit the antioxidant and antiproliferative effect of TA methanolic extract against a variety of human cancer cells (16,18,19). Owing to possess promising biological activities against various ailments in different cellular and animal models, our preliminary published work documents the antiproliferative activity of TA methanolic extract against tumor cell models (breast and colorectal). The current study for the first time demonstrated that TA methanolic extract of dry leaves exhibits cytotoxic potential and promotes simultaneous induction of autophagy and apoptosis in HCC (HepG2) cells. A mechanistic study reveals that upon treatment with varying dose of TA methanolic extract, HCC (HepG2) cells augment autophagic cell death by promotes the formation of autophagosomes which were analyzed by acridine orange (AO) staining. Further, the immunoblotting demonstrates the steady conversion of microtubule-associated protein 1A/1B-light chain 3-I (LC3I) to microtubule-associated protein 1A/1B-light chain 3-II (LC3II) with gradual degradation of Sequestosome-1 (SQSTM1/p62) upon treatment with varying doses of TA. Moreover, we observed a significant induction of apoptosis which was supported by activation of apoptosis marker proteins caspase-3 and PARP1 at higher doses of TA methanolic extract. DAPI staining, phase contrast microscopy, and elevated levels of ROS further confirmed the cell killing by apoptosis. Additionally, TA methanolic extract triggers cell cycle arrest at the G0/G1 phase and increases the expression of cell cycle checkpoint proteins p27/Kip and p21/Cip with a decrease in expression of cyclin D1 in cells treated with varying doses of TA methanolic extract (Fig. 1). Together these results suggest that the medicinal plant TA can be explored for potential drug candidate molecules to curtail cancer in the future.

Previous reports demonstrated that numerous plant extracts exhibit an antiproliferative potential against a wide range of cellular models of cancer (33,34). TA methanolic extract reveals several pharmacological properties like anti-inflammatory, hepatoprotective, anti-oxidative, and antiproliferative activity by modulates miR-1275 in hepatocellular carcinoma cells (18,19). Thus, we sought to examine the in vitro inhibitory activity of TA methanolic extract against the cell proliferation of HCC cells (HepG2, Huh7, Hep3B, and THLE-2). We observe that TA methanolic extract starts attenuation of cellular growth kinetics of HCC (HepG2, Huh7, Hep3B, and THLE-2) cells at an incredibly low dose of 10 µg/mL. It was observed that the IC<sub>50</sub> value range is (271±4.38), (298±7.08) and (336±6.11) µg/mL against HepG2, Huh7D12, and Hep3B cell lines, respectively (Fig. 2 and Table 1). Interestingly, when the inhibitory effect on the growth of transformed normal THLE-2 cells was evaluated, we observe the 50 % population of cells were died at (3641±23.74 µg/mL) of TA methanolic extract.
extract (Fig. 2 and Table 1), which is significantly a quite high dose of TA methanolic extract compared to IC_{50} dose of HCC (HepG2, Huh7, Hep3B, and THLE-2) cells, indicates that TA methanolic extract not only curtails the cellular growth kinetics of HCC cells specifically but also displays safe cytotoxicity potential against normal transformed cells. Together, our results suggest that the methanolic extract of dry leaves of halophytic plant TA displays potential cytotoxic effect against a panel of HCC cells and leaving normal transformed liver cells with fewer cytotoxic effect.

Next, the microscopic analysis indicated that a substantial number of dead, floating tumor cells were observed at higher doses of TA methanolic extract than untreated control (Fig. 3a), indicates that the activation of cell death pathways in TA treatment groups compared to DMSO control. Further, cell killing by TA methanolic extract was also supported by the nuclear morphology DAPI stain, which reveals the change in membrane blebbing and nuclear morphology, which are the hallmarks of apoptosis (Fig. 3b). To corroborate, the above results demonstrate that the TA methanolic extract induces a potent antiproliferative effect on the cellular models of HCC (HepG2, Huh7, Hep3B, and THLE-2).

Badmus et. al. suggest that *Holarrhena floribunda* extract displayed an antiproliferative activity against tumor cells, triggers cell cycle arrest by inducing ROS-mediated apoptosis induction (35). Similarly, Kowalczyk et. al. showed that *Menyanthes trifoliata* L. extract significantly inhibits the growth of glioma cells by attenuating the expression of anti-apoptotic proteins, triggers arrest of the G2/M phase of cell cycle and activates mitochondrial-dependent apoptosis and augments the expression of apoptosis proteins Bax and caspase-3 (36). Various studies have revealed that natural compounds obtained from plants have the promising apoptosis-inducing ability in cancer cells (37–39).

Mechanistically, apoptosis induced by natural compounds disrupts the association of anti-apoptotic protein Bcl-2 from pro-apoptotic proteins thereby activates a cascade of pro-apoptotic proteins (40). Plant-based extracts and derived compounds have been documented to activate cell death mechanisms by activating cellular caspases, promotes intrinsic apoptosis by releasing cytochrome C, induces ROS production, and activation of AMPK which ultimately leads to activate cell death mechanisms in tumor cells (41,42). Thus, we want to evaluate the apoptotic potential of TA methanolic extract against HCC (HepG2) cells. To evaluate the apoptosis potential of TA methanolic extract in HCC cells, we expose HepG2 cells to different doses of TA methanolic extract for 24 h along with untreated control. By flow cytometry, we observe a substantial cellular population of HepG2 cells undergoes apoptosis (23.1 %) at a higher dose of TA methanolic extract (500 μg/mL) when compared with positive control staurosporine (25 nM) (42.1 %) (Fig. 4a and 4b). To further confirm the activation of apoptosis, we perform immunoblotting analysis of HepG2 cells treated with TA methanolic extract in a dose-dependent manner (125, 250, and 500 μg/mL). Our results suggest that higher doses of TA
methanolic extract activate apoptosis promoting proteins by induces prominent cleavage of caspase-3 and PARP1 when compared to untreated control (Fig. 4c).

ROS and associated peroxidases play a key role in the regulation of cell death in multiple cancer cell types. Accumulated evidence revealed that elevation of ROS by natural products is the major driver of cancer cells towards the activation of cell death pathways. In the recent past, numerous reports suggest that chemotherapeutic agents induce apoptotic cell death by elevating ROS production (26). To examine whether TA methanolic extract could also induce ROS production in HepG2 cells, we expose HepG2 cells to varying doses of TA in presence of ROS activator H₂O₂ (positive control, 100 μM) along with untreated control for 24 h and stain the cells with DCFHDA dye after the treatment. Owing to pass easily through membrane, DCFHDA dye is disintegrated by cellular stress and catalyses a reaction with peroxidases to form green, fluorescent DCF (dichlorofluorescein). Intriguingly, we observed TA promotes significant ROS product ion in a concentration-dependent manner as compared to untreated control. However, HepG2 cells exposed to positive control (H₂O₂, 100 μM) significantly promote ROS production (Fig. 5a and 5b). Together, these results demonstrate that TA methanolic extract activates apoptosis mediated proteins and promotes ROS production which might have a critical role in the augmentation of apoptosis.

Autophagy is one of the important catabolic physiological processes that help to clear the old and damaged cellular contents to recycle metabolites with the help of lysosomes and utilize them during energy crises. Autophagy plays a dual role in cancer therapy and tumorigenesis. Accumulating evidence suggests that most natural products that exhibit antiproliferative activity induce autophagic cell death mode in tumor cells (22). The previous report revealed that in hepatocellular carcinoma cells, berberine suppresses hepatocellular carcinoma cells, by induces simultaneously mitochondrial-mediated apoptosis and autophagic cell death (42). Numerous reports suggest that plant-based natural compounds and extracts induces autophagy and kills tumor cells by various cell death mechanisms (43,44). Therefore, we sought to reveal whether TA methanolic extract treatment could augment the induction of autophagy in HepG2 cells. To evaluate the HepG2 cells were plated overnight and exposed to varying doses of TA methanolic extract as depicted in Fig. 6a. After completion of incubation for 24 h, the cells were stained with acridine orange (AO) staining. As shown in Fig. 6b and 6c, although, many cells in lower doses of TA methanolic extract could display acidic vesicular organelles (AVOs), however, cells exposed to higher doses of TA methanolic extract has significant induction of prominent AVOs after quantification and appears bright red in the cytoplasm of HepG2 cells. Further, immunoblotting of HepG2 cells at higher doses of TA methanolic extract revealed the prominent conversion of LC3B-I to LC3B-II, which indicates the maturation of autophagosome and induction of autophagy (Fig. 6c). To further evaluate the effect of TA methanolic
extract on the flux of autophagy in HepG2 cells, we observe the reduced expression with the low band intensity of sequestrosome 1 (P62) protein, which is a specific substrate of autophagy, at higher doses of TA methanolic extract compared to lower doses. Collectively, these results suggest that HepG2 cells exposed to TA methanolic extract stimulates autophagic flux and augments cell death in tumor cells.

Accumulating evidence suggests that numerous phytochemicals derived from natural sources have been reported to exhibit antiproliferative activity by triggering cell cycle arrest thereby promotes cell death via apoptosis (44,45). Tumor cell proliferation is primarily controlled by the cell cycle (46), which consists of four distinct progressive phases (subG1/G0, G1, S, and G2/M) (47). Under normal circumstances, the progression of the cell cycle is tightly regulated by a cascade activation of cyclins, cyclin-dependent kinases (CDKs), and cyclin-dependent kinase inhibitors. However, in malignant cells, aberrant expression of cell cycle regulators attenuates the differentiation of cells that results in irregular cell growth. In the recent past, numerous reports suggest that natural compounds having antiproliferative activity by triggering cell cycle arrest. Luo et al. (48) demonstrated that 6-Gingerol (6-G) enhances the radiosensitivity of gastric cancer cells by the induction of apoptosis and cell cycle arrest (48). Additionally, Lin et al. (49) reveal that 6-G displays suppression of cell motility of colon cancer cells induces ROS, and upregulates p21/Cip, p27/Kip thereby arrests cell cycle in colon cancer cells (49). To examine whether TA methanolic extract could arrest the cell cycle, HepG2 cells were synchronized in a low serum-containing medium, HepG2 cells were progressed through various cell cycle phases in a serum-containing medium for at least 3 h. The cells were then treated with varying doses (125, 250, and 500 µg/mL) of TA methanolic extract for 24 h. Our results demonstrated that TA methanolic extract arrests the cell cycle at G0/G1 phase in HepG2 cells. The quantification of cells at various cell cycle phases showed that a significant population of cells (18.1 %) was halted at the G0/G1 phase of the cell cycle when HepG2 cells were exposed to 500 µg/mL TA as compared to untreated control (3.6 %) and positive control camptothecin 2 µM (25.4 %) (Fig. 7a and 7b). Next, we sought to examine the effect of TA methanolic extract in the cell cycle regulatory proteins. Our immunoblotting results demonstrate a drastic reduction in cyclin D1 expression and a subsequent increase in the expression of p21/cip1 and p27/Kip1 proteins when HepG2 cells were exposed to higher doses of TA methanolic extract (Fig. 7c). Collectively these results demonstrate that TA methanolic extract induced G0/G1 phase cell cycle arrest and increases the expression of cell cycle checkpoint proteins p27/Kip1 and p21/Cip1 with a concomitant decrease in expression of cyclin D1 in HepG2 cells.

CONCLUSIONS
Our results for the first-time document that TA methanolic extract of dry leaves exhibits cytotoxic activity and simultaneous activation of both the cell-death associated processes: apoptosis and autophagy in HepG2 cells. Mechanistically, TA induces autophagy which was confirmed by prominent orange red color to acidic autophagosomes and steady conversion of LC3I to LC3II with gradual degradation of SQSTM1/p62. Further, TA methanolic extract induces ROS-mediated apoptosis which was confirmed by activation of apoptosis marker proteins caspase-3 and PARP1. Additionally, TA methanolic extract triggers the arrest of the G0/G1 phase in the cell cycle and increases the expression of cell cycle checkpoint proteins p27/Kip and p21/Cip with a decrease in expression of cyclin D1 in cells treated with varying doses of TA methanolic extract. Collectively, these results prove that the medicinal plant TA can be explored for potential drug candidate molecules to curtail cancer.

DECLARATION OF COMPETING INTEREST

The authors declare that they have no conflict of interest.

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AUTHORS’ CONTRIBUTION

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Table 1. Represents the IC₅₀/(μg/mL) value of TA methanolic extract of dry leaves against a panel of HCC cells (HepG2, Huh-7D12, Hep3B) along with transformed normal hepatic cells (THLE-2). Data presented were performed three or more than three times and the mean value of ±SE, *p<0.05

<table>
<thead>
<tr>
<th>S. No</th>
<th>Cell line</th>
<th>IC₅₀/(μg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>HepG2</td>
<td>271±4.38</td>
</tr>
<tr>
<td>2</td>
<td>Huh-7 D12</td>
<td>298±7.08</td>
</tr>
<tr>
<td>3</td>
<td>Hep3B</td>
<td>336±6.11</td>
</tr>
<tr>
<td>4</td>
<td>THLE-2</td>
<td>3641±23.74</td>
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</table>
Fig. 1. The schematic diagram showed the methanolic extract of dry leaves of TA exerts the antiproliferative effect by simultaneous induction of cell death by two biological conserved processes autophagy by the maturation of LC3I to LC3II followed by the degradation SQSTM1/P62 and apoptosis which is associated with activation caspase-3 and PARP1 cleavage in HepG2 cells. Further, the TA methanolic extract of dry leaves triggers cell cycle arrest at G0/G1 phase of the cell cycle by upregulating the cell cycle regulatory proteins p27/Kip and p21/Cip with concomitant reduction in cyclin D1 in HCC cells.
Fig. 2. TA methanolic extract of dry leaves inhibits cell proliferation of HCC (HepG2, Huh-7D12, Hep3B, and transformed normal cell line THLE-2) cells. a) TA methanolic extract of dry leaves promotes antiproliferative effect against HCC cells (HepG2, Huh-7D12, Hep3B, and transformed normal cell line THLE-2) by the MTT assay. Data presented were performed three or more than three times and the mean value of ±SE, *p<0.05
Fig. 3. TA methanolic extract of dry leaves induces cell killing in HepG2 cells. a) HepG2 cells treated with varying doses of TA methanolic extract of dry leaves in the presence of positive control (staurosporine 25 nM) and untreated control (DMSO). The presented images were taken by a microscope inbuilt with a camera after HepG2 cells were treated with indicated doses of TA methanolic extract of dry leaves. Scale bar: 100 µm; 20x, b) Represents the DAPI staining images after treated with indicated doses of TA methanolic extract of dry leaves along with positive control camptothecin (2 µM) and untreated control (DMSO). Scale bar: 50 µm; 20x
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Fig. 4. TA methanolic extract of dry leaves promotes the induction of apoptosis in HepG2 cells. a) Quantification of the apoptotic population by flow cytometry of HepG2 cells after 24 h treatment with different doses of TA methanolic extract of dry leaves along with positive control (staurosporine 25 nM) and untreated control (DMSO). b) The histogram represents the percentage of apoptotic HepG2 cells of the above-mentioned experiment. c) Protein expression of apoptosis activated proteins.
(caspase-3, PARP1 along with β-actin as an internal control) of HepG2 cells after 24 h treatment with different doses of TA methanolic extract of dry leaves along with positive control (staurosporine 25 nM) and untreated control (DMSO). Data presented were performed three or more than three times and the mean value of ±SE, **p<0.01
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**Fig. 5.** TA methanolic extract of dry leaves induces ROS production in HepG2 cells. a) Fluorescent images of DCFDA staining of HepG2 cells After 24 h treatment with different doses of TA methanolic extract of dry leaves along with untreated control (DMSO), and positive control (H₂O₂). Scale bar: 100 μm; 20x. b) The fluorescence intensity of TA methanolic extract of dry leaves, positive control (H₂O₂), and control (DMSO) treated DCF cells was quantified by a microplate reader, in 3 independent experiments. Data presented were performed three or more than three times and the mean value of ±SE, *p<0.05
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Fig. 6. TA methanolic extract of dry leaves induces autophagy in HepG2 cells. a) Representative microphotographs of HepG2 cells captured by fluorescent microscope display the acidic vesicular formation or autophagosome formation stained by acridine orange (1 mg/mL) after 24 h treatment with different doses of TA methanolic extract of dry leaves along with positive control (rapamycin 100 nM) and untreated control (DMSO). The magnification is 20x and scale bar 100 µm. b) The histogram represents the percentage of acridine orange positive HCC cells upon treatment with different doses
of TA methanolic extract of dry leaves along with positive control (rapamycin 100 nM) and untreated control (DMSO). c) Expression of autophagy proteins LC3B-I to LC3B-II conversion and p62/SQSTM1 expression along with β-actin as an internal control in HCC cells upon 24 h treatment with different doses of TA methanolic extract of dry leaves along with positive control (rapamycin 100 nM) and untreated control (DMSO). Data presented were performed three or more than three times and the mean value of ±SE, *p<0.05
Fig. 7. TA methanolic extract of dry leaves induces G0/G1 phase cell cycle arrest and modulates cell cycle regulatory proteins in HepG2 cells. a) Quantification of cells in different stages of the cell cycle by flow cytometry after 24 h treatment of HepG2 cells with different doses of TA methanolic extract of dry leaves is subjected to cell cycle analysis along with positive control (camptothecin 2 μM) and untreated control (DMSO). b) The bar diagram presents the quantification of G0/G1 phase cell cycle arrest in HepG2 cells after 24 h treatment with different doses of TA methanolic extract of dry leaves along with positive control (camptothecin, 2 μM) and untreated control (DMSO). c) Immunoblotting analysis of HepG2 cells exposed to varying doses of TA methanolic extract of dry leaves along with positive control (camptothecin, 2 μM) and untreated control (DMSO) shows the expression profile of cell cycle regulation proteins (cyclin D1, p21/Cip1, and p27/Kip1) using β-actin as an internal control. Data presented were performed three or more than three times and the mean value of ±SE, *p<0.05