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Original Article

Evaluation of *in vitro* anticancer potential of pharmacological ethanolic plant extracts *Acacia modesta* and *Opuntia monocantha* against liver cancer cells

Avaliação do potencial anticancerígeno in vitro de extratos etanólicos de plantas farmacológicas *Acacia modesta* e *Opuntia monocantha* contra células cancerígenas do fígado

F. Abid[a,b], M. Saleem[a,c], T. Leghari[d], I. Rafi[d], T. Maqbool[e*], F. Fatima[d], A. M. Arshad[d], S. Khurshid[b], S. Naz[f], F. Hadi[e], M. Tahir[e], S. Akhtar[e,g], S. Yasir[h], A. Mobashar[b] and M. Ashraf[e]

[a]Government College University Faisalabad, Faculty of Pharmaceutical Sciences, Faisalabad, Pakistan
[b]The University of Lahore, Faculty of Pharmacy, Lahore, Pakistan
[c]University of the Punjab, Punjab University College of Pharmacy, Lahore, Pakistan
[d]University of Lahore, University College of Medicine and Dentistry, Lahore, Pakistan
[e]University of Lahore, Institute of Molecular Biology and Biotechnology, Centre of Research in Molecular Medicine, Lahore, Pakistan
[f]University of Lahore, Faculty of Allied Health Sciences, Lahore, Pakistan
[g]University of Bradford, School of Medical Sciences, Bradford, United Kingdom
[h]University of Lahore, Department of Math and Statistics, Lahore, Pakistan

Abstract

Acacia modesta (AM) and *Opuntia monocantha* (OM) are distributed in Pakistan, Afghanistan and India. Both of these plants have different pharmacological properties. This study was designed to evaluate anticancer potential of *Acacia modesta* (AM) and *Opuntia monocantha* (OM). Liver cancer cell line HepG2 was used for assessment of anticancer activity. For the evaluation of anti-proliferative effects, cell viability and cell death in all groups of cells were evaluated via MTT, crystal violet and trypan blue assays. For the evaluation of apoptosis ELISA of p53 performed. Furthermore, LDH assay to find out the ability of malignant cells to metabolize pyruvate to lactate and antioxidant enzymes activity (GSH, CAT and SOD) at the end HPLC was performed to find active compound of AM and OM. Cytotoxicity (MTT), Viability assays (trypan blue, crystal viability, MUSE analysis) showed more dead, less live cells in plant treated groups with increase of concentration. Scratch assay for the anti-migratory effect of these plants showed treated groups have not ability to heal scratch/wound. ELISA of p53 for cellular apoptosis showed more release of p53 in treated groups. Antioxidant assay via glutathione (GSH), superoxide dismutase (SOD), catalase (CAT) showed less anti-oxidative potential in treated cancer groups. LDH assay showed more lactate dehydrogenase release in treated groups compared with untreated. HPLC analysis showed the presence of phytochemicals such as steroids, alkaloids, phenols, flavonoids, saponins, tannins, anthraquinone and amino acids in AM and OM plant extracts. Based on all these findings, it can be concluded that ethanolic extracts of *Acacia modesta* and *Opuntia monocantha* have promising anti-cancer potential.

Keywords: *Acacia modesta*, *Opuntia monocantha*, liver cancer, HepG2 cells, cell viability, scratch assay, ELISA p53, LDH, GSH, SOD, CAT, HPLC.

Resumo

Acacia modesta (AM) e *Opuntia monocantha* (OM) estão distribuídas no Paquistão, Afeganistão e Índia. Ambas as plantas têm propriedades farmacológicas diferentes. Este estudo foi desenhado para avaliar o potencial anticancerígeno de *Acacia modesta* (AM) e *Opuntia monocantha* (OM). A linha celular de câncer de fígado HepG2 foi usada para avaliação da atividade anticâncer. Para a avaliação dos efeitos antiproliferativos, a viabilidade celular e a morte celular em todos os grupos de células foram avaliadas através dos ensaios de MTT, cristal violeta e azul de tripano. Para a avaliação da apoptose foi realizado ELISA de p53. Além disso, o ensaio de LDH para descobrir a capacidade das células malignas de metabolizar o piruvato em lactato e a atividade das enzimas antioxidantes (GSH, CAT e SOD) no final da HPLC foi realizado para encontrar o composto ativo de AM e OM. Citotoxicidade (MTT), ensaios de viabilidade (azul de tripano, viabilidade de cristal, análise MUSE) mostraram mais células mortas e menos células vivas nos grupos tratados com plantas com aumento da concentração. O ensaio de arranhão para o efeito anti-migratório dessas plantas mostrou que os grupos tratados não têm capacidade de cicatrizar arranhão/ferida.

*e-mail: tahir.maqbool@imbb.uol.edu.pk
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ELISA de p53 para apoptose celular mostrou maior liberação de p53 nos grupos tratados. Ensaio antioxidante via glutationa (GSH), superóxido dismutase (SOD), catalase (CAT) mostrou menor potencial antioxidante nos grupos de câncer tratados. O ensaio de LDH mostrou mais liberação de lactato desidrogenase nos grupos tratados em comparação com os não tratados. A análise de HPLC mostrou a presença de fitoquímicos como esteróides, alcalóides, fenóis, flavonóides, saponinas, taninos, antraquinona e aminoácidos nos extratos vegetais AM e OM. Com base em todos esses achados, pode-se concluir que os extratos etanólicos de *Acacia modesta* e *Opuntia monocantha* apresentam potencial anticancerígeno promissor.

Palavras-chave: *Acacia modesta*, *Opuntia monocantha*, câncer de fígado, células HepG2, viabilidade celular, ensaio de arranhão, ELISA p53, LDH, GSH, SOD, CAT, HPLC.

1. Introduction

Natural plants are rich source of medicine all over the world due to their less side effects. World Health organization mentioned these herbal medicines as primary health care in various regions of the world (El-Wahab et al., 2013). This is evident that just about seventy-four antitumor compounds are either natural product or natural product derived (Chen et al., 2004; Cragg and Newman, 2005; Tan et al., 2006).

Phytochemicals are gaining importance in pharmacological studies nowadays. *Acacia modesta* [AM] and *Opuntia monocantha* [OM] plant extracts have been shown to possess anti-inflammatory effect (Bukhari et al., 2010; Yang and Meng, 2008). AM is found in the tropical areas of India and Pakistan (Punjab, Khyber Pakhtunkhwa and Baluchistan) (Baloch et al., 2017). Various parts of AM have been used to prepare different extracts and showed pharmacological activities including anti-platelet, anti-inflammatory (Sokeng et al., 2013) and hypoglycemic (Rahaman and Chaudhry, 2015). OM, on the other hand, is cactus specie, found in the tropical and subtropical regions. It can be grown in the areas having less vegetative properties. It has been used traditionally due to its pharmacological effectiveness in case of burns, diabetes (Yang and Meng, 2008), indigestion and bronchial asthma. OM cladodes show pharmacological effect due to presence of certain polysaccharides (Zhao et al., 2007; Bari et al., 2012). The anti-inflammatory effect of flavonoid components of OM is also reported in mice (He et al., 2011). AM and OM have shown to poses anticancer activity against cervical cancer cells (Abid et al., 2020). Conventional treatments of cancer such as chemotherapy and radiation therapy help in diminishing the bulk of cancer cells but the overall population remains same. The true therapeutic strategy is to focus on natural plants and phytochemicals for eradicating the cancer.

Cancer is considered as a fatal disease globally. Primary liver cancer is the fourth cause of cancer-related mortality worldwide. With more than 750,000 new cases annually (33,000 in the United States), it has become the fastest growing malignancy in the world, both in terms of incidence and mortality (Losic et al., 2020). The number of new cases is estimated to be 564,000 per year, including 398,000 in men and 166,000 in women. In high-risk countries, liver cancer can arise before the age of 20 years, whereas, in countries at low risk, liver cancer is rare before the age of 50 years (Bosch et al., 2004). The morbidity and mortality rates vary widely and reflect the uneven distribution of the main risk factors. In most areas with a high incidence of liver cancer, the main risk factors are chronic hepatitis B virus [HBV] infection and consumption of food contaminated with the mycotoxin, aflatoxin B1. Conversely, in most low-incidence areas, the main risk factors are hepatitis C virus [HCV] infection, excessive alcohol consumption, obesity, and diabetes. The International Agency for Research on Cancer [IARC] classifies HBV and HCV as carcinogenic to humans. Hepatocellular carcinoma (HCC) is associated with a very poor prognosis (Thorgeirsson and Grisham, 2002) and the third most common cause of death worldwide. Oftenly, liver fibrosis is associated with HCC which can lead to severe cirrhosis at advanced stage. Therefore, the prognosis of liver cancer depends on the history of liver disease (Ghany et al., 2003) due to accumulation of reactive oxygen species in organ during fibrotic state and hepatocytes become injured in oxidative stress (Sakurai et al., 2013). Thus there is a need for economical, effective and safe alternatives for treatment of the disease.

Therefore, the aim of current study was to evaluate the *in-vitro* cytotoxic activity of ethanolic extracts of AM and OM against HepG2 liver cancer cell line.

2. Methods

2.1. Preparation of extracts

Preparation of extracts was done via cold maceration method according to Maqbool T (Maqbool et al., 2019).

2.2. Culturing of cell line

The cryovials obtained from liquid nitrogen cylinder were revived and cultured in T-75 culturing flask supplemented with DMEM-high glucose medium (Gibco) and fetal bovine serum (10% FBS) (Gibco), and penicillin and streptomycin (10^6 per Litre) (Gibco). When cultured HepG2 cells reached 70-80% confluency there sub-culturing was performed. For splitting, PBS was used for washing and trypsin/EDTA for detachment and then observed under an inverted microscope. After detachment, cells were centrifuged and plated in 96, 24 and 6 well plates for further assays.

2.3. Treatment

Treatment was divided into 3 groups (triplicate) 1st group untreated 2nd groups ethanolic extract AM treated HepG2 cells and 3rd group ethanolic extract OM treated HepG2 cells. Further 96 well plate for MTT, trypan blue, crystal violet, Antioxidants, LDH, p53 ELISA and 6 well plate for MUSE analysis, Scratch assay.

2.4. MTT assay

IC50 was evaluated via 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay [18]. Briefly, cells were cultured and treated for 72 hours treatment. Monolayer of cells was first washed with phosphate buffer saline (PBS) (Invitrogen Inc., USA), further cells were incubated in 100 µl complete medium containing 25 µl MTT solution (Invitrogen Inc., USA) for 2 hrs. MTT converted into purple colored formazan in living cells which was then solubilized with dimethyl sulphoxide (DMSO) (Invitrogen Inc., USA) and absorbance of solution was taken at 570 nm. AM and OM plants extracts were applied with 0.25 mg/mL, 0.5 mg/mL, 1 mg/mL, 2 mg/mL & 3 mg/mL concentrations.

2.5. Trypan blue assay

IC50 value of AM and the maximum cytotoxicity concentration of OM extract were applied for cell viability was assessed using trypan blue as prohibiting agent for live and dead cells. The cells of different experimental groups were washed with PBS three times leading to incubation in trypan blue (Invitrogen Inc., USA) for 15 min. Cells were then washed with PBS three times and observed under microscope. Stained cells with trypan blue were considered as dead. Percentage was calculated by dividing cells without staining with total number of cells and multiplying by 100 (Maqbool et al., 2019).

2.6. Crystal violet assay

IC50 value of AM and the maximum cytotoxicity concentration of OM extract were applied for cell viability which was assessed by crystal violet staining method done on HepG2 cell line using an already established protocol (Maqbool et al., 2019). This method was conducted in a 96-well plate. Medium from different experimental groups was discarded from wells of plate and washed with PBS. After washing 0.1% crystal violet dye mixed with 2% ethanol was added on the wells in a way that surface was covered. It was incubated at room temperature for 15 minutes. Dye was discarded carefully, and wells were washed with extensive care so that cells may not lift off from the well. Then 600 µl of 1% SDS was added in each well to solubilize the stain and left for 5-10 minutes. At the end, absorbance was taken at 540 nm or 595 nm on microtiter plate.

2.7. Scratch assay

Scratch assay was performed in 6 well cell culture plate by using the IC50 values of extracts following Liang et al. protocol (Liang et al., 2007). Images were captured at 0, 48 and 72 hours.

2.8. Muse analysis via count and viability kit

IC50 value of plant AM and maximum cytotoxicity concentration of OM were applied on HepG2 cells which were cultured in 6 well culture plates by using the Muse™ automated cell analyzer (Merck-Millipore) via count and viability kit (Cat. No MCH100102). After 72 hours of treatment cells were centrifuged for 5 minutes at 2000 RPM, supernatant was discarded and cell pellet was re-suspended in cell and viability reagent followed by the counting of cells as mentioned in Hadi et al. (2020).

2.9. Evaluation of antioxidant enzymes: Glutathione Reductase (GSH), Superoxide Dismutase (SOD) and Catalase (CAT) assays

GSH, SOD and CAT assays were performed in a 96-well plate with a reaction mixture of 200µl in each well according to Maqbool et al., Liang et al. and Zhou et al. protocols (Maqbool et al., 2019; Liang et al., 2007; Zhou et al., 2009). The absorbance was taken at 340 nm, 560 nm and 240 nm respectively and values were plotted on the graph.

2.10. Lactate dehydrogenase assay (LDH)

The activity of LDH assay was measured in the supernatant medium taken from all experimental groups in 96 well plates and 5 µl medium from each experimental group along with 100 µl of working reagent for 5 min. Absorbance was measured by spectrophotometer at 340 nm.

2.11. Enzyme linked immunosorbent assay (ELISA)

Solid phase sandwich ELISA was performed for p53 (Santa Cruz Biotechnology, USA) in a 96-well plate (Corning, USA) according to protocol established by (Maqbool et al., 2019). Solid phase sandwich ELISA was performed for p53 (Santa Cruz Biotechnology, USA) in a 96-well plate (Corning, USA). Capture antibody i.e. rabbit polyclonal anti p53 antibody (Santa Cruz biotechnology, USA) was diluted in a coating buffer to a concentration of 2-10 µg/ mL. 100 µl of this diluted antibody was then transferred to each well of the plate and incubated for 120 minutes. After the incubation, the capture antibody was discarded from each well and then washed. Each well was washed three times with 1X TBS-T (washing solution) for three minutes. Then, 200 µl of blocking solution (BSA) was added for 30 minutes. After that blocking solution was removed. Add 200 µl of culturing medium harvested from different experimental groups to each well and after 30 minutes medium was removed, and each well was washed three times. After washing, 100 µl of secondary antibody HRP conjugated donkey anti-rabbit secondary antibody (Santa Cruz biotechnology, USA) was added to each well and the plate was incubated for 60 minutes. After incubation, secondary antibody was removed, and each well was washed three times. For HRP detection, Tetramethylbenzidine (TMB) (Invitrogen Inc., USA) is the most popular chromogenic substrate. 100 µl of TMB was added (chromogenic substrate) to each well then, the plate was incubated for 20 minutes. After incubation, 100 µl of stop solution 0.18 M sulphuric acid (H_2SO_4) was added to stop the TMB reaction, absorbance was taken at 450 nm by using the microtiter plate.

2.12. HPLC

Major bioactive compounds (flavonoids and phenolics) in AM and OM were derived through HPLC. A 10 mg weight of both extracts was added in 5 mL of deionized water, along with 12 mL of ethanol for sample preparation. An already reported standard method was used as described by

Sultana et al. (2008). Ultraviolet visible detector was used for the detection of compounds at 280 nm.

2.13. Statistical analysis

All experiments were performed in triplicate. Data were analyzed by Graph Pad Prism 5 (Graph Pad Software, Inc., CA, USA). All data were represented as means and standard deviation (SD), whereas statistical comparisons were made using one way analysis of variance (ANOVA) with Bonferroni compare, where $p \leq 0.05$ was considered as the minimal level of significance.

3. Results

3.1. MTT assay for IC50 and cytotoxicity

IC50 of AM and cytotoxicity of OM were observed through MTT assay which is a reliable method for measuring cell viability. Cell viability of plants extracts on HepG2 cell line is expressed as percentage cell viability.

Ethanolic AM extract was screened for its cytotoxicity against HepG2 liver cancer cells with increasing concentrations to determine the IC50 value. The IC50 value was found to be 0.37 mg/mL. In comparison, the control samples did not showed any significant effect on cell growth. The ethanolic extract of OM also showed concentration-dependent cytotoxicity against HepG2 liver cancer cells. Extract with concentration of 2 mg/mL showed highest toxic potential. AM ethanolic extracts showed slightly enhanced cytotoxicity as compared with the extracts of OM in a concentration dependent manner. Combined concentrations of ethanolic extracts of AM and OM on HepG2 liver cancer cells exhibited synergic effect and exhibited enhanced toxicity potential than individual extracts (Figure 1 and Table 1).

3.2. Trypan blue (dead cells detection) cell viability assay

Cells viability was assessed by trypan blue (dead cells detection) assay for detection and evaluation of dead as shown in Table 2. HepG2 cancer cells were treated with AM and OM extracts and staining with trypan blue. A significantly large number of blue colored cells were

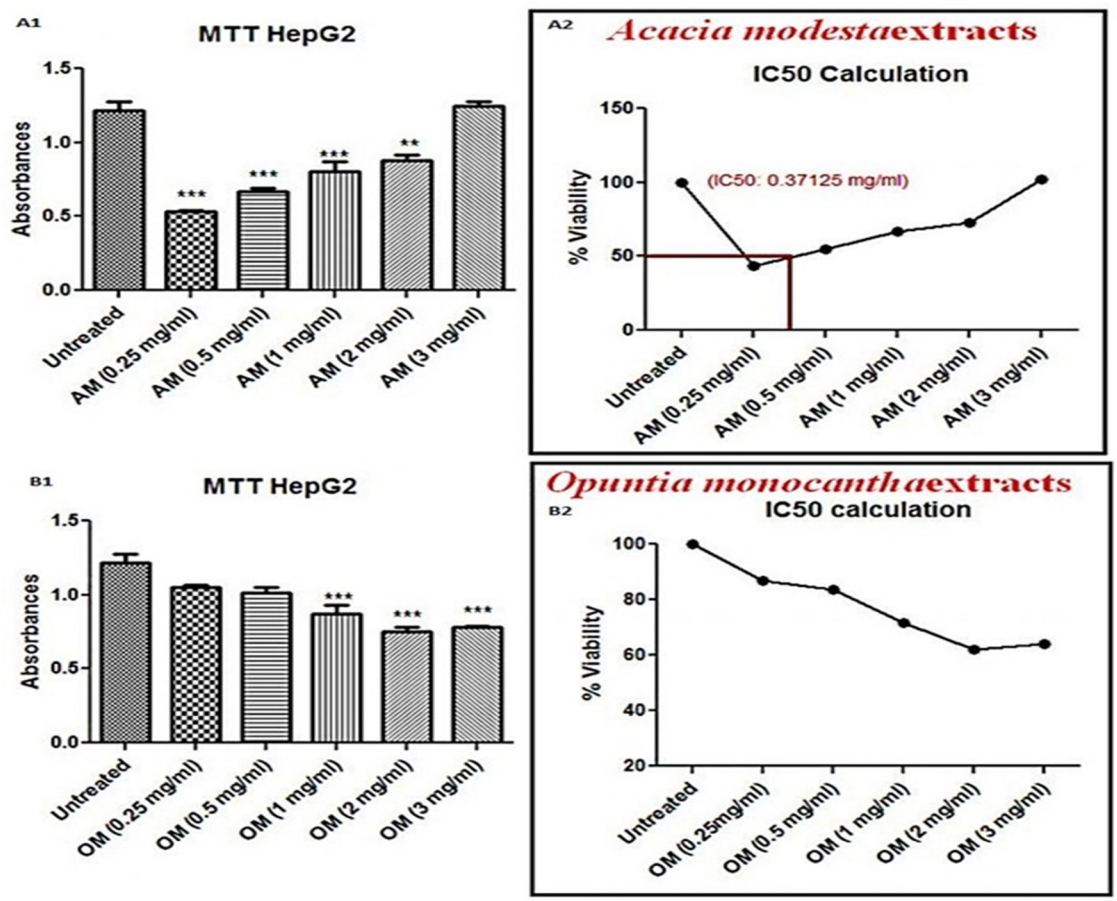

Figure 1. Cytotoxicity assay and IC50 values of *Opuntia monocantha* and *Acacia modesta* extracts on HepG2 cell line using MTT assay. Where A1, A2 are HepG2 treated with AM extracts B1, B2 are HepG2 treated with OM extracts, whereas UT is untreated, AM (0.25 mg/mL, 0.5 mg/mL, 1 mg/mL, 2 mg/mL and 3 mg/mL) and OM (0.25 mg/mL, 0.5 mg/mL, 1 mg/mL, 2 mg/mL and 3 mg/mL). Data are representative of 3 experiments, mean ± SD.

observed in AM and OM treated HepG2 cells results indicated more dead cells as compared to the untreated HepG2 cells. (Figure 2).

3.3. Crystal violet (live cells detection) cell viability assay

Cells viability was further evaluated by crystal violet (live cells detection) assay for detection and evaluation of live cells as shown in Table 2. In crystal violet assay (live cells detection) treated HepG2 cells showed less live cells as compared to the untreated cells based upon the absorbance (Figure 3).

3.4. Muse analysis via count and viability kit

HepG2 cells treated with AM and OM extracts had higher cytotoxic effect when compared to the untreated group. In case of untreated group, 96.6% cells were viable, whereas treatment with ethanolic extract of AM represented 58.8% and treatment with ethanolic extract of OM represented 66.2% viable cells. The results of cell count and viability are shown in Figure 4.

3.5. Decreased antioxidants level in treated HepG2 groups

The antioxidant potential using the catalase (CAT), superoxide dismutase (SOD) and glutathione reductase

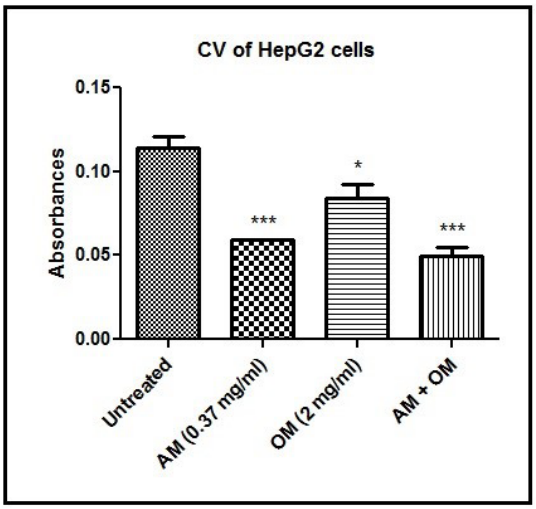

Figure 2. Cell viability analysis of extracts on HepG2 cells via crystal violet analysis (live cells detection). According to figure, IC50 concentration of AM (0.37 mg/mL) and maximum cytotoxicity concentration of OM (2 mg/mL) and combination of AM (0.37 mg/mL) + OM (2 mg/mL). *, *** showing significance difference between untreated and treated groups.

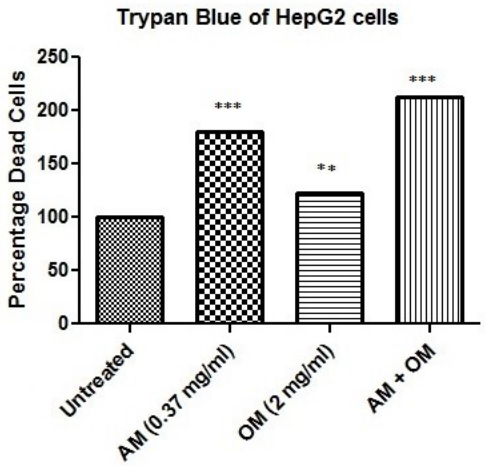

Figure 3. Cell viability analysis of extracts on HepG2 cells via trypan blue analysis (dead cells detection). According to figure, IC50 concentration of AM (0.37 mg/mL) and maximum cytotoxicity concentration of OM (2 mg/mL) and combination of AM (0.37 mg/mL) + OM (2 mg/mL). **, *** showing significance difference between untreated and treated groups.

Table 1. IC50 of AM and OM extracts against HepG2 cell line.

Untreated [Mean ± SD]	AM0.25 mg/mL	AM	AM	AM	AM
		0.5 mg/mL	1 mg/mL	2 mg/mL	3 mg/mL
1.21 ± 0.10	0.53 ± 0.01	0.66 ± 0.03	0.80 ± 0.11	0.88 ± 0.06	1.2 ± 0.05
Untreated	**OM**	**OM**	**OM**	**OM**	**OM**
[Mean ± SD]	**0.25 mg/mL**	**0.5 mg/mL**	**1 mg/mL**	**2 mg/mL**	**3 mg/mL**
1.2 ± 0.10	1.05 ± 0.021	1.01 ± 0.06	0.86 ± 0.10	0.75 ± 0.04	0.78 ± 0.02
Collective AM+OM extracts					
Untreated [Mean ± SD]			**AM + OM**		
1.21 ± 0.102			0.54 ± 0.046		

Table 2. Effects of AM, OM and AM+OM extracts on cell viability in trypan blue and crystal violet viability assays.

Assays	Untreated [Mean ± SD]	AM [Mean ± SD]	OM [Mean ± SD]	AM + OM [Mean ± SD]
Trypan blue	100 ± 0.00	18 ± 1.53	129 ± 5.52	213 ± 4.51
Crystal violet cell	0.11 ± 0.01	0.60 ± 0.01	0.08 ± 0.01	0.50 ± 0.01

(GSH) assays which determines the free radical scavenging activity. It was observed that GSH, CAT, and SOD activities were decreased in treated groups as compared to untreated groups shown in Table 3 and Figure 5.

3.6. More lactate dehydrogenase levels in treated HepG2 cells

Moreover, high LDH release was evaluated in treatment group of HepG2 cells compared to the untreated groups Figure 6 and Table 4.

3.7. More p53 release in treated HepG2 cells

p53 ELISA was performed for evaluating the apoptosis level by the use of p53 antibody, which is a principal apoptotic related protein. Table 4 and Figure 7 showed that the AM and OM treatment groups exhibited higher level of apoptosis as compared to the untreated group.

3.8. Scratch assay

According to the results, treatment of extracts after the scratch limited the growth of cells and prevented them to heal the wound as compared to the untreated cells. Results showed that untreated cells were able to repair while in case of treatment with AM and OM cells lost the repairing capacity Figure 8.

3.9. Multiple phenolic and flavonoids compounds

HPLC analysis of extracts revealed the presence of multiple phenolic and flavonoids phytochemicals such as, steroids, alkaloids, saponins, tannins, anthraquinone and amino acids in AM and OM extracts Figure 9.

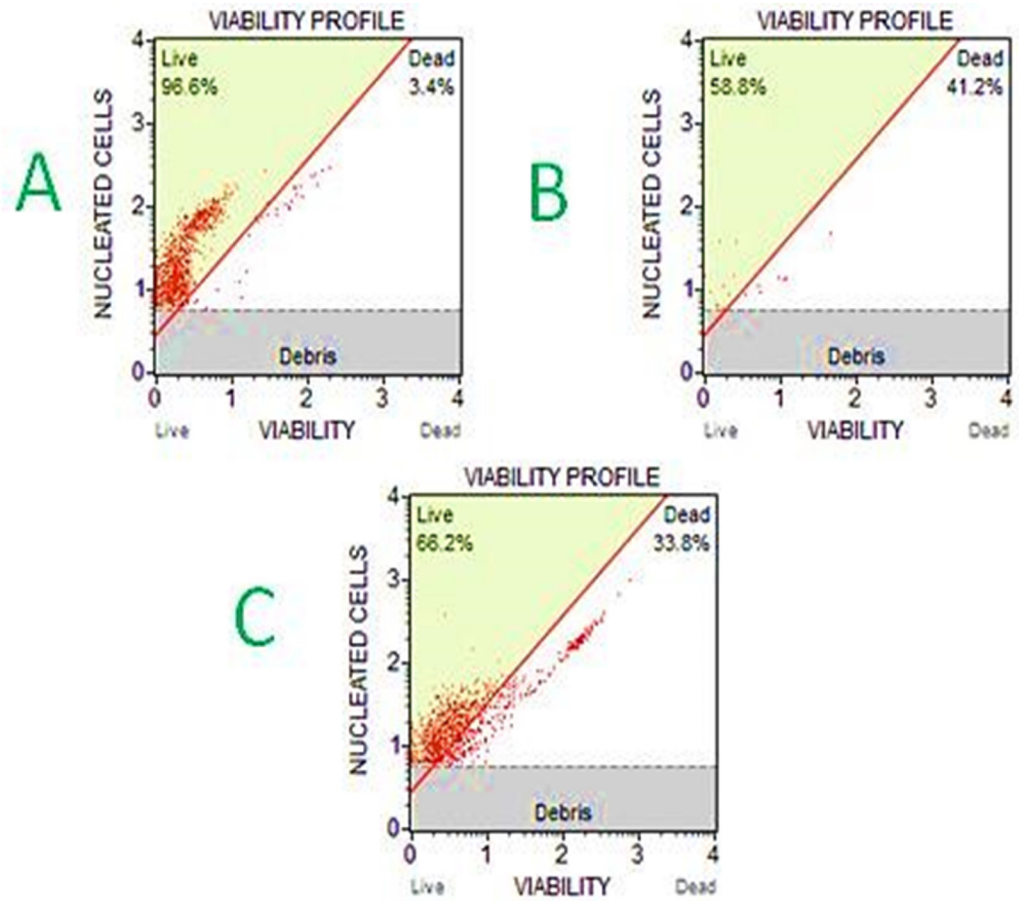

Figure 4. MUSE analysis of HepG2 cells via count and viability kit where A is untreated showing 96.6% live cells, B is AM showing 58.8% live cells and C is OM showing 66.2% live cells.

Table 3. Antioxidant potential of AM, OM and AM+OM extracts.

Antioxidants	Untreated [Mean ± SD]	AM [Mean ± SD]	OM [Mean ± SD]	AM+OM [Mean ± SD]
SOD	1.14±0.0871	0.660±0.0515	0.916±0.0825	0.568±0.0867
CAT	1.07±0.00917	0.611±0.0372	0.814±0.0866	0.566±0.0465
GSH	1.04±0.0170	0.549±0.0226	0.742±0.00920	0.554±0.0661

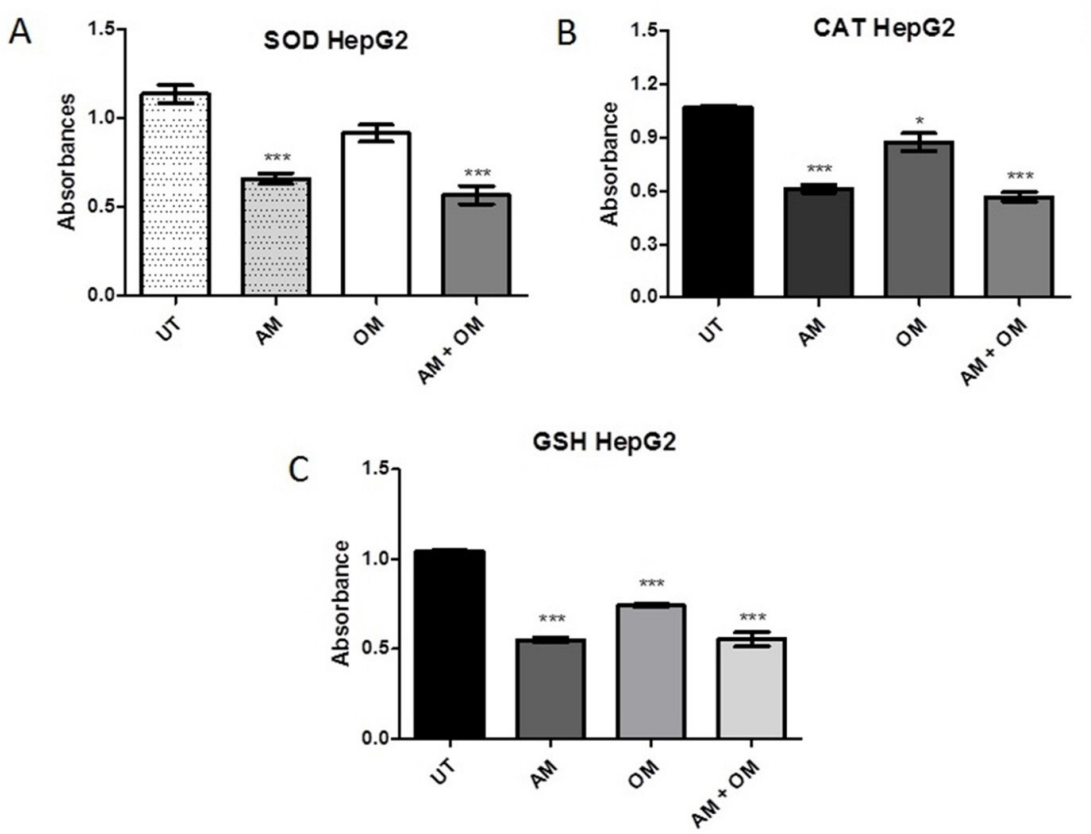

Figure 5. Antioxidative evaluation where A is superoxide dismutase, B is catalase and C is glutathione. According to figure, IC50 concentration of AM (0.37 mg/mL) and maximum cytotoxicity concentration of OM (2 mg/mL) and combination of AM (0.37 mg/mL) + OM (2 mg/mL). *, *** showing significance difference between untreated and treated groups.

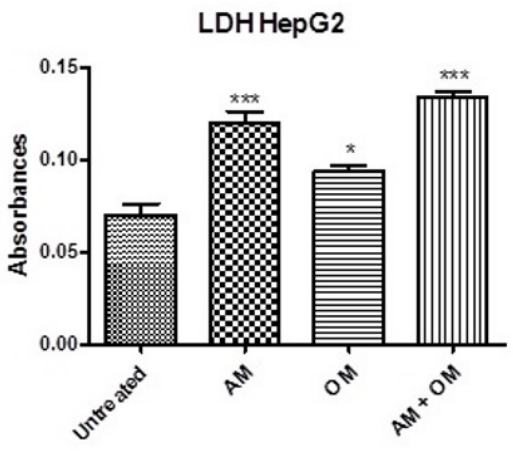

Figure 6. Analysis of lactate dehydrogenase More LDH released was evaluated in treatment group of HepG2 cells compared with untreated groups. According to figure, IC50 concentration of AM (0.37 mg/mL) and maximum cytotoxicity concentration of OM (2 mg/mL) and combination of AM (0.37 mg/mL) + OM (2 mg/mL). *, *** showing significance difference between untreated and treated groups.

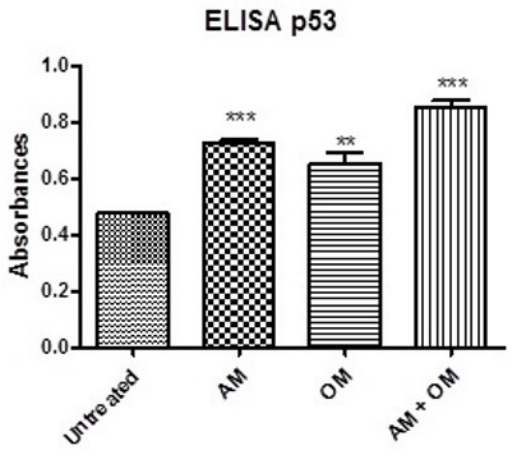

Figure 7. Apoptosis evaluation with p53 ELISA. Where more release of p53 was observed in case of all three treatment groups as compared with untreated groups. According to figure, IC50 concentration of AM (0.37 mg/mL) and maximum cytotoxicity concentration of OM (2 mg/mL) and combination of AM (0.37 mg/mL) + OM (2 mg/mL). **, *** showing significance difference between untreated and treated groups.

Table 4. Effect of AM, OM and AM+OM extracts on p53 and LDH release.

Assays	Untreated [Mean ± SD]	AM [Mean ± SD]	OM [Mean ± SD]	AM + OM [Mean ± SD]
p53	0.475 ± 0.00608	0.727 ± 0.0168	0.650 ± 0.0705	0.853 ± 0.0401
Lactate dehydrogenase [LDH]	0.070 ± 0.010	0.120 ± 0.0100	0.0933 ± 0.00577	0.133 ± 0.00577

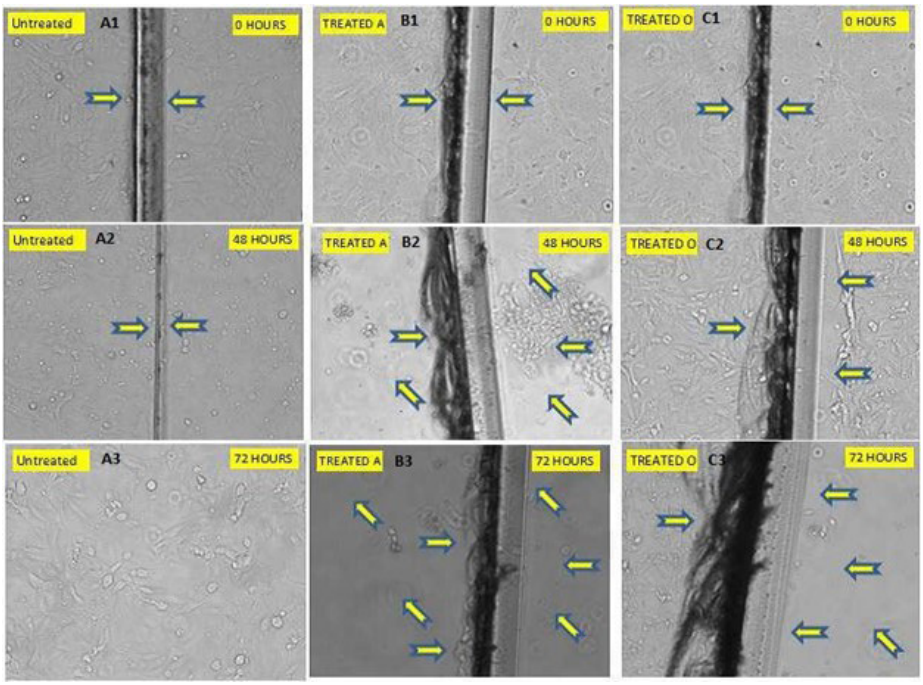

Figure 8. Effects of different treatments on the migration of HepG2 cells images were captured after 0 hour, 24 hours and 72 hours of treatment. Where A1-A3 are untreated groups with 0 hour, 48 hours and 72 hours, B1-B3 are untreated groups with 0 hour, 48hr and 72hrs, C1-C3 are untreated groups with 0 hour, 48 hours and 72 hours. Arrows are indicating scratch in case of untreated cells, whereas, scratch, ruptured and damaged cells in case of treated cells.

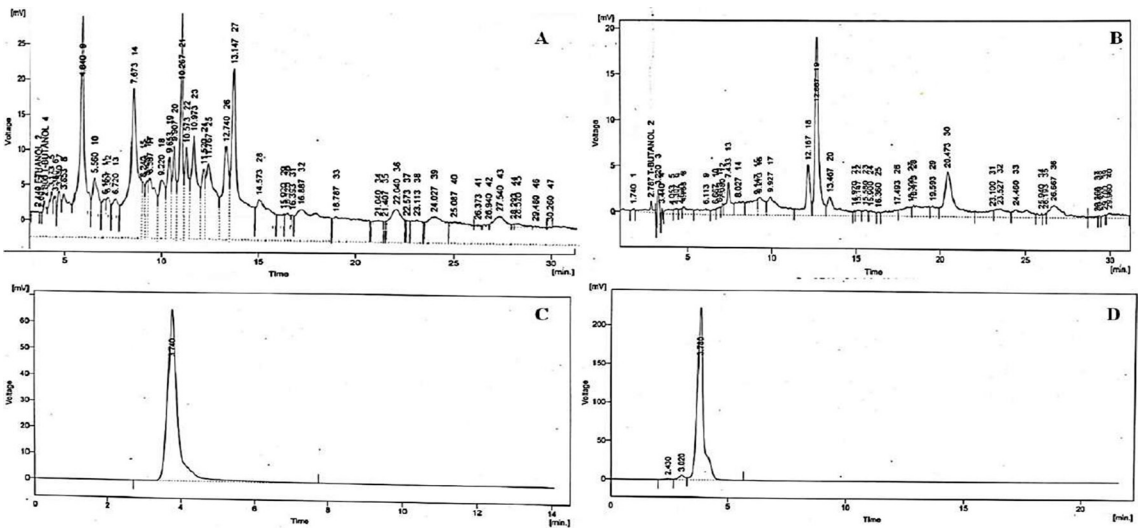

Figure 9. HPLC (High performance liquid chromatography) chromatogram of ethanolic extract of AM and OM. **(A)** showing quercetin, benzoic acid, gallic acid, vanillic acid, sinapic acid, ferulic acid, syringic acid and cinnamic acid and **(B)** showing quercetin, benzoic acid, gallic acid, vanillic acid, sinapic acid, M-coumaric acid, syringic acid and Pcoumaric acid and **(C)** showing HPLC chromatogram of vitamin C compound of AM and **(D)** showing HPLC chromatogram of vitamin C compound of OM.

4. Discussion

Many plants have been evaluated for their different pharmacological activities all over the world. Many cases have been reported in which plants containing common generic name, showed similar properties. Similarly, *Acacia nilotica* (Sakthivel et al., 2012) and *Acacia hydaspica* (Afsar et al., 2016) have been reported to exhibit anticancer activity. In the current study, another plant with same generic name and different specie name, *Acacia modesta* (AM) was evaluated for its anticancer potential against the liver cancer cells. Traditionally, this plant was used to treat various diseases such as backache, bacterial infections, venereal diseases, cough, dysentery, wound healing, and leprosy. Similarly, its antidiabetic property has also been reported. Although its aqueous and n-hexane extracts did not display any cytotoxic effect (Saleem et al., 2018), satisfactory results were found when treated with ethanolic extract. Similarly, related genus members of OM also displayed anticancer activity such as *Opuntia ficus-indica* (Abou-Elella and Ali, 2014) and *Opuntia hemifusa* (Hahm et al., 2015). Cancer is considered as the second largest disease in the world with the multi-mechanistic mechanisms. Hence, it requires a multidimensional approach for its control, prevention, and the cure. In therapeutic world, plant based drugs play a vital role in treating various human ailments. In the past few decades, use of phytochemicals in cancer prevention has received much interest. Many plants derived anticancer agents are available in the market such as camptothecin, podophyllotoxin, taxanes, vinca alkaloids and their derivatives. Similarly, many plants derived agents are under evaluation to find out their anticancer potential such as homoharringtonine, 4-ipomeanol and β-lapachone. In the current study, OM and AM were evaluated for their anticancer potential against the liver cancer.

MTT assay is one of the most versatile and popular assay for cytotoxicity dependent on the conversion of substrate to chromogenic product by live cells. The MTT assay involves the conversion of the water-soluble yellow dye MTT [3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide] to an insoluble purple formazan by the action of mitochondrial reductase (Kumar et al., 2018). Activity of both plants was assessed by MTT via application of different concentrations i.e., 0.25 mg/mL, 0.5 mg/mL, 1 mg/mL, 2 mg/mL and 3 mg/mL, where plant AM showed better results at optimum concentration (IC50) of 0.371 mg/mL and in case of OM at concentration of 2 mg/mL showed better results (Figure 1).

Moreover, trypan blue exclusion test was used to determine the number of viable cells present in a cell suspension. It is based on the principle that live cells possess intact cell membranes that exclude certain dyes, such as trypan blue, eosin, or propidium, whereas dead cells do not. In this test, a cell suspension is simply mixed with dye and then visually examined to determine whether cells take up or exclude dye (Strober, 2015). In current study, evaluation of cell viability by the trypan blue (dead cell detection) method showed an increased trend in plant AM and OM extracts compared with the untreated cells, particularly with treated combination of both AM and OM plant extracts. The increased percentage of dead cells suggested that AM and OM, and also the combination of AM + OM treatment disrupted cell membrane of liver cancer cells and was very effective against hepatocellular carcinoma. Furthermore, crystal violet staining being a quick and versatile assay for screening cell viability under diverse stimulation conditions (Geserick et al., 2009). However, it is potentially compromised by proliferative responses that occur at the same time as cell death responses. Therefore, chemical inhibitors of caspases and/or of necroptosis may be incorporated into the assay (Degterev et al., 2008; Sun et al., 2012). Alternatively, molecular studies (e.g., overexpression or knockdown) can be performed to more specifically address the nature of cell death (Feoktistova et al., 2011). Evaluation of cell viability by the crystal violet method showed a decreasing trend in treated HepG2 cells, especially with treated combination of AM + OM. The decreased percentage of viable cells suggesting that plant AM and plant OM, also combination of AM + OM treatment are very effective against hepatocellular carcinoma as there are less viable cells in treatment group as compared to the untreated group (Figures 2-3).

Count and viability assay is a fluorescent-based analysis using Muse analyzer, which is more specific and reliable method to quantify the number of viable cells. The DNA binding dyes present in the reagent differentially stain viable and non-viable cells based on their permeability and provide an accurate count of both the cells (Jose et al., 2018). According to current study, less live cells were observed in treated groups (Figure 4).

Oxidative stress is the primary marker of cancer. Antioxidant enzymes such as GSH, CAT and SOD affect the cell proliferation in a positive way. But when anti proliferative and antioxidants are combined for cancer therapy, they improve the anticancer effect (Lendahl et al., 2009; George et al., 2015). In the current study, when cells were treated with AM and OM, the antioxidative level of GSH, SOD and CAT were significantly decreased in treated groups compared to the untreated groups (Figure 5).

LDH measurement is very simple and reliable for verification of cytotoxicity assay. In case of in-vitro trials when cell membrane is damaged due to endogenous or exogenous factors, LDH is released and can be estimated in the supernatants of cell cultures. Many studies showed that LDH level did not increase in untreated cells while treated cells showed high levels of LDH (George et al., 2015; Saad et al., 2006). In current study, plant extracts showed the same result, cells with the treatment of plant extracts showed high level of LDH release in comparison with the untreated cells (Figure 6).

Apoptosis induction is a useful strategy for anticancer drug development. Plant derived anti-cancer drugs exert cell death by inducing apoptosis in cancer cells. Many mechanisms responsible for apoptosis induced by plants and most of them induce apoptotic cell death by intrinsic or extrinsic pathways and p53 dependent or independent pathway. In current findings, it was observed that plant extracts of AM and OM induced apoptosis in HepG2 cells via p53 dependent pathway (Figure 7).

One of the few cell migration assays, which can even be performed without video-microscopy, since the closure of the wound can be estimated at fixed time points. Several hours after wounding, directional collective migration is easily assessed and quantified (Jose et al., 2018) in the cancer cells. It is best to prevent scratch to heal after treatment. In the present study, after the scratch given to cells along with treatment of plant extracts AM + OM, cells were totally unable to repair in comparison with untreated group where cells proliferated and with passage of time from day 0 to 72 hours, the scratch was repaired (Figure 8).

The phytochemical analysis of both plant extracts has indicated the existence of flavonoids and phenols like quercetin, gallic acid, vanllic acid, caffeic acid, benzoic acid, chlorogenic acid, syringic acid, cinamic acid and sinapic acid. Thus, plant active compounds like quercetin, gallic acid, ferulic acid, coumaric acid and benzoic acid are reported to possessed remarkable anti-oxidant, anti-inflammatory and anticancer activities (Hazafa et al., 2020). While *Opuntia monocantha* was devoid of ferulic acid and additionally contained P. coumaric and N. coumaric acid. Moreover, both plants also contained vitamin C compound. HPLC analysis of both plant extracts showed the presence of P-coumaric acid that is a good candidate as anti-inflammatory agent. The presence of sinapic acid in both plants extract makes them a worthy candidate for anti-inflammatory and antiarthritic drug alternative. Sinapic acid exerts anti-inflammatory effect by inhibiting the COX-2 and proinflammatory cytokines. Gallic acid is polyphenol found in both plant extracts and anticancer effect of gallic acid is reported previously as it inhibits cancer cell proliferation and induces apoptosis. Anticancer effect of gallic acid is due to inhibition of NF-Kb pathway. It also revealed the presence of multifunctional fatty acids include vitamin E in ethanolic extract of *Acacia modesta* that has been immensely documented as antioxidant and anti-platelet agent (Hazafa et al., 2020). Presence of different compounds like eucalyptol, beta sitosterol, vitamin E that have antioxidant, anti-cancer and anti-inflammatory activities. Eucalyptol has anti-inflammatory activity and antioxidant activity by regulating NF-κβ and MAPK pathway in multiple chronic ailments. It is also involved in reducing the pulmonary inflammation by lowering the TNF-alpha, IL-1b and NF- κβ level 9 (Seol and Kim, 2016). Beta sitosterol is a dietary phytosterol has a strong anti-cancer activity against many cancers (Kumar et al., 2016). Linalyl acetate is also found which is recognized as anti-inflammatory and anti-oxidant agent (Peana et al., 2002). Additionally, Beta sitosterol is plant derived nutrient found in ethanolic extract of *Acacia modesta* having anticancer property against different cancers. Many studies showed that beta sitosterol interfere with the multiple cell signaling pathways together with apoptosis survival and inflammation. Due to presence of such phytochemicals in the ethanolic extract, *Acacia modesta* shown significant result against cancer and inflammation (Sayeed and Ameen, 2015). Conclusively, being superfluous with all these compounds, which might be the reason both extracts of test substances showed promising anti-arthritic and anticancer activity (Figure 9). These compounds display a remarkable spectrum of biological activities and due to these properties, these extracts were able to dysregulate cancer development.

5. Conclusion

This study elaborated an anticancer potential of *Acacia modesta* and *Opuntia monocantha* plants which can be further evaluated by studying the active components and their chemotherapeutic properties to be screened at further levels such as animal trials as a possible anticancer treatment.

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References

ABID, F., SALEEM, M., MULLER, C.D., ASIM, M.H., ARSHAD, S., MAQBOOL, T. and HADI, F., 2020. Anti-proliferative and apoptosis-inducing activity of Acacia Modesta and Opuntia Monocantha extracts on HeLa cells. *Asian Pacific Journal of Cancer Prevention*, vol. 21, no. 10, pp. 3125-3131. http://dx.doi.org/10.31557/APJCP.2020.21.10.3125. PMid:33112576.

ABOU-ELELLA, F.M. and ALI, R.F.M., 2014. Antioxidant and anticancer activities of different constituents extracted from Egyptian prickly pear cactus [Opuntia Ficus-Indica] peel. *Biochemistry and Analytical Biochemistry*, vol. 3, no. 2, p. 1. http://dx.doi.org/10.4172/2161-1009.1000158.

AFSAR, T., RAZAK, S., KHAN, M.R., MAWASH, S., ALMAJWAL, A., SHABIR, M. and HAQ, I.U., 2016. Evaluation of antioxidant, anti-hemolytic and anticancer activity of various solvent extracts of Acacia hydaspica R. Parker aerial parts. *BMC Complementary and Alternative Medicine*, vol. 16, no. 1, p. 258. http://dx.doi.org/10.1186/s12906-016-1240-8. PMid:27473625.

BALOCH, A.H., BALOCH, I.A., REHMAN, H.-U., AHMED, I. and AHMED, S., 2017. A study of poisonous plants of Balochistan, Pakistan. *Pure and Applied Biology*, vol. 6, no. 3, pp. 989-1001. http://dx.doi.org/10.19045/bspab.2017.600105.

BARI, M.N., ZUBAIR, M., RIZWAN, K., RASOOL, N., BUKHARI, I.H., AKRAM, S., BOKHARI, T.H., SHAHID, M., HAMEED, M. and AHMAD, V.U., 2012. Biological activities of Opuntia Monacantha cladodes. *Journal of the Chemical Society of Pakistan*, vol. 34, no. 4, pp. 990-995.

BOSCH, F.X., RIBES, J., DÍAZ, M. and CLÉRIES, R., 2004. Primary liver cancer: worldwide incidence and trends. *Gastroenterology*, vol. 127, no. 5, suppl. 1, pp. S5-S16. http://dx.doi.org/10.1053/j.gastro.2004.09.011. PMid:15508102.

BUKHARI, I.A., KHAN, R.A., GILANI, A.H., AHMED, S. and SAEED, S.A., 2010. Analgesic, anti-inflammatory and anti-platelet activities of the methanolic extract of Acacia modesta leaves. *Inflammopharmacology*, vol. 18, no. 4, pp. 187-196. http://dx.doi.org/10.1007/s10787-010-0038-4. PMid:20390366.

CHEN, M.S., CHEN, D. and DOU, Q.P., 2004. Inhibition of proteasome activity by various fruits and vegetables is associated with cancer cell death. *In Vivo*, vol. 18, no. 1, pp. 73-80. PMid:15011755.

CRAGG, G.M. and NEWMAN, D.J., 2005. Plants as a source of anti-cancer agents. *Journal of Ethnopharmacology*, vol. 100, no.

1-2, pp. 72-79. http://dx.doi.org/10.1016/j.jep.2005.05.011. PMid:16009521.

DEGTEREV, A., HITOMI, J., GERMSCHEID, M., CH'EN, I.L., KORKINA, O., TENG, X., ABBOTT, D., CUNY, G.D., YUAN, C., WAGNER, G., HEDRICK, S.M., GERBER, S.A., LUGOVSKOY, A. and YUAN, J., 2008. Identification of RIP1 kinase as a specific cellular target of necrostatins. *Nature Chemical Biology*, vol. 4, no. 5, pp. 313-321. http://dx.doi.org/10.1038/nchembio.83. PMid:18408713.

EL-WAHAB, A.E.A., GHAREEB, D.A., SARHAN, E.E., ABU-SERIE, M.M. and DEMELLAWY, M.A.E., 2013. In vitro biological assessment of Berberis vulgaris and its active constituent, berberine: antioxidants, anti-acetylcholinesterase, anti-diabetic and anticancer effects. *BMC Complementary and Alternative Medicine*, vol. 13, no. 1, p. 218. http://dx.doi.org/10.1186/1472-6882-13-218. PMid:24007270.

FEOKTISTOVA, M., GESERICK, P., KELLERT, B., DIMITROVA, D.P., LANGLAIS, C., HUPE, M., CAIN, K., MACFARLANE, M., HÄCKER, G. and LEVERKUS, M., 2011. cIAPs block ripoptosome formation, a RIP1/caspase-8 containing intracellular cell death complex differentially regulated by cFLIP isoforms. *Molecular Cell*, vol. 43, no. 3, pp. 449-463. http://dx.doi.org/10.1016/j.molcel.2011.06.011. PMid:21737330.

GEORGE, B.P.A., TYNGA, I.M. and ABRAHAMSE, H., 2015. In vitro antiproliferative effect of the acetone extract of Rubus fairholmianus gard. Root on human colorectal cancer cells. *BioMed Research International*, vol. 2015, p. 165037. PMid:26078938.

GESERICK, P., HUPE, M., MOULIN, M., WONG, W.W.-L., FEOKTISTOVA, M., KELLERT, B., GOLLNICK, H., SILKE, J. and LEVERKUS, M., 2009. Cellular IAPs inhibit a cryptic CD95-induced cell death by limiting RIP1 kinase recruitment. *The Journal of Cell Biology*, vol. 187, no. 7, pp. 1037-1054. http://dx.doi.org/10.1083/jcb.200904158. PMid:20038679.

GHANY, M.G., KLEINER, D.E., ALTER, H., DOO, E., KHOKAR, F., PROMRAT, K., HERION, D., PARK, Y., LIANG, T.J. and HOOFNAGLE, J.H., 2003. Progression of fibrosis in chronic hepatitis C. *Gastroenterology*, vol. 124, no. 1, pp. 97-104. http://dx.doi.org/10.1053/gast.2003.50018. PMid:12512034.

HADI, F., AWAN, S.J., TAYYEB, A., MAQBOOL, T., SHEHZADI, S., MALIK, S., KAUSAR, H. and MALIK, A., 2020. Hepato-protective role of itraconazole mediated cytochrome p450 pathway inhibition in liver fibrosis. *Pakistan Journal of Pharmaceutical Sciences*, vol. 33, suppl. 6, pp. 2751-2758. PMid:33879433.

HAHM, S.-W., PARK, J., OH, S.-Y., LEE, C.-W., PARK, K.-Y., KIM, H. and SON, Y.S., 2015. Anticancer properties of extracts from Opuntia humifusa against human cervical carcinoma cells. *Journal of Medicinal Food*, vol. 18, no. 1, pp. 31-44. http://dx.doi.org/10.1089/jmf.2013.3096. PMid:25379883.

HAZAFA, A., REHMAN, K.U., JAHAN, N. and JABEEN, Z., 2020. The role of polyphenol (flavonoids) compounds in the treatment of cancer cells. *Nutrition and Cancer*, vol. 72, no. 3, pp. 386-397. http://dx.doi.org/10.1080/01635581.2019.1637006. PMid:31287738.

HE, K., CHEN, X., WAN, Y. and LI, Y., 2011. Study on the anti-inflammatory effect of total flavonoids from Opuntia monacantha [Willd.] Haw. *Chinese Veterinary Science*, vol. 41, no. 10, pp. 1065-1069.

JOSE, G.M., RAGHAVANKUTTY, M. and KURUP, G.M., 2018. Sulfated polysaccharides from Padina tetrastromatica induce apoptosis in HeLa cells through ROS triggered mitochondrial pathway. *Process Biochemistry*, vol. 68, pp. 197-204. http://dx.doi.org/10.1016/j.procbio.2018.02.014.

KUMAR, P., NAGARAJAN, A. and UCHIL, P.D., 2018. Analysis of cell viability by the MTT assay. *Cold Spring Harbor Protocols*, vol. 2018, no. 6, p. pdb.prot095505. http://dx.doi.org/10.1101/pdb.prot095505. PMid:29858338.

KUMAR, V.A., AMMANI, K., JOBINA, R., PARASURAMAN, P. and SIDDHARDHA, B., 2016. Larvicidal activity of green synthesized silver nanoparticles using *Excoecaria agallocha* L. (Euphorbiaceae) leaf extract against *Aedes aegypti*. *IET Nanobiotechnology*, vol. 10, no. 6, pp. 382-388. http://dx.doi.org/10.1049/iet-nbt.2015.0101. PMid:27906138.

LENDAHL, U., LEE, K.L., YANG, H. and POELLINGER, L., 2009. Generating specificity and diversity in the transcriptional response to hypoxia. *Nature Reviews. Genetics*, vol. 10, no. 12, pp. 821-832. http://dx.doi.org/10.1038/nrg2665. PMid:19884889.

LIANG, C.-C., PARK, A.Y. and GUAN, J.-L., 2007. In vitro scratch assay: a convenient and inexpensive method for analysis of cell migration in vitro. *Nature Protocols*, vol. 2, no. 2, pp. 329-333. http://dx.doi.org/10.1038/nprot.2007.30. PMid:17406593.

LOSIC, B., CRAIG, A.J., VILLACORTA-MARTIN, C., MARTINS-FILHO, S.N., AKERS, N., CHEN, X., AHSEN, M.E., VON FELDEN, J., LABGAA, I., D'AVOLA, D., ALLETTE, K., LIRA, S.A., FURTADO, G.C., GARCIA-LEZANA, T., RESTREPO, P., STUECK, A., WARD, S.C., FIEL, M.I., HIOTIS, S.P., GUNASEKARAN, G., SIA, D., SCHADT, E.E., SEBRA, R., SCHWARTZ, M., LLOVET, J.M., THUNG, S., STOLOVITZKY, G. and VILLANUEVA, A., 2020. Intratumoral heterogeneity and clonal evolution in liver cancer. *Nature Communications*, vol. 11, no. 1, p. 291. http://dx.doi.org/10.1038/s41467-019-14050-z. PMid:31941899.

MAQBOOL, T., AWAN, S.J., MALIK, S., HADI, F., SHEHZADI, S. and TARIQ, K., 2019. In-vitro anti-proliferative, apoptotic and antioxidative activities of medicinal herb kalonji [Nigella sativa]. *Current Pharmaceutical Biotechnology*, vol. 20, no. 15, pp. 1288-1308. http://dx.doi.org/10.2174/1389201020666190821144633. PMid:31433749.

PEANA, A.T., D'AQUILA, P.S., PANIN, F., SERRA, G., PIPPIA, P. and MORETTI, M.D., 2002. Anti-inflammatory activity of linalool and linalyl acetate constituents of essential oils. *Phytomedicine*, vol. 9, no. 8, pp. 721-726. http://dx.doi.org/10.1078/094471102321621322. PMid:12587692.

RAHAMAN, M.S. and CHAUDHRY, M.A., 2015. Evaluation of antioxidant and hepatoprotective effect of Acacia modesta wall against paracetamol induced hepatotoxicity. *Journal of Pharmaceutical Research International*, vol. 5, no. 5, pp. 336-343. http://dx.doi.org/10.9734/BJPR/2015/15559.

SAAD, B., DAKWAR, S., SAID, O., ABU-HIJLEH, G., BATTAH, F.A., KMEEL, A. and AZIAZEH, H., 2006. Evaluation of medicinal plant hepatotoxicity in co-cultures of hepatocytes and monocytes. *Evidence-Based Complementary and Alternative Medicine*, vol. 3, no. 1, pp. 93-98. http://dx.doi.org/10.1093/ecam/nel002. PMid:16550229.

SAKTHIVEL, K.M., KANNAN, N., ANGELINE, A. and GURUVAYOORAPPAN, C., 2012. Anticancer activity of Acacia nilotica [L.] Wild. Ex. Delile subsp. indica against Dalton's ascitic lymphoma induced solid and ascitic tumor model. *Asian Pacific Journal of Cancer Prevention*, vol. 13, no. 8, pp. 3989-3995. http://dx.doi.org/10.7314/APJCP.2012.13.8.3989. PMid:23098505.

SAKURAI, T., KUDO, M., UMEMURA, A., HE, G., ELSHARKAWY, A.M., SEKI, E. and KARIN, M., 2013. p38α inhibits liver fibrogenesis and consequent hepatocarcinogenesis by curtailing accumulation of reactive oxygen species. *Cancer Research*, vol. 73, no. 1, pp. 215-224. http://dx.doi.org/10.1158/0008-5472.CAN-12-1602. PMid:23271722.

SALEEM, B., ISLAM, M., SAEED, H., IMTIAZ, F., ASGHAR, M., SALEEM, Z., MEHMOOD, A. and NAHEED, S., 2018. Investigations of Acacia modesta Wall. leaves for in vitro anti-diabetic, proliferative and cytotoxic effects. *Brazilian Journal of Pharmaceutical*

Sciences, vol. 54, no. 2, p. e17467. http://dx.doi.org/10.1590/s2175-97902018000217467.

SAYEED, M.S.B. and AMEEN, S.S., 2015. Beta-sitosterol: a promising but orphan nutraceutical to fight against cancer. *Nutrition and Cancer*, vol. 67, no. 8, pp. 1216-1222. http://dx.doi.org/10.1080/01635581.2015.1087042. PMid:26473555.

SEOL, G.H. and KIM, K.Y., 2016. Eucalyptol and its role in chronic diseases. In: S. GUPTA, S. PRASAD and B. AGGARWAL, eds. *Drug discovery from mother nature*. Cham: Springer, Advances in Experimental Medicine and Biology, vol. 929, pp. 389-398. http://dx.doi.org/10.1007/978-3-319-41342-6_18.

SOKENG, S.D., KOUBÉ, J., DONGMO, F., SONNHAFFOUO, S., NKONO, B.L.N.Y., TAÏWÉ, G.S., CHERRAH, Y. and KAMTCHOUING, P., 2013. Acute and chronic anti-inflammatory effects of the aqueous extract of Acacia nilotica [L.] Del. [Fabaceae] pods. *Academia Journal of Medicinal Plants*, vol. 1, no. 1, pp. 001-005.

STROBER, W., 2015. Trypan blue exclusion test of cell viability. *Current Protocols in Immunology*, vol. 111, no. 1, pp. A3.B.1-A3.B.3. http://dx.doi.org/10.1002/0471142735.ima03bs111. PMid:26529666.

SULTANA, B., ANWAR, F., ASI, M.R. and CHATHA, S.A.S., 2008. Antioxidant potential of extracts from different agro wastes: stabilization of corn oil. *Grasas y Aceites*, vol. 59, no. 3, pp. 205-217.

SUN, L., WANG, H., WANG, Z., HE, S., CHEN, S., LIAO, D., WANG, L., YAN, J., LIU, W., LEI, X. and WANG, X., 2012. Mixed lineage kinase domain-like protein mediates necrosis signaling downstream of RIP3 kinase. *Cell*, vol. 148, no. 1-2, pp. 213-227. http://dx.doi.org/10.1016/j.cell.2011.11.031. PMid:22265413.

TAN, G., GYLLENHAAL, C. and SOEJARTO, D.D., 2006. Biodiversity as a source of anticancer drugs. *Current Drug Targets*, vol. 7, no. 3, pp. 265-277. http://dx.doi.org/10.2174/138945006776054942. PMid:16515527.

THORGEIRSSON, S.S. and GRISHAM, J.W., 2002. Molecular pathogenesis of human hepatocellular carcinoma. *Nature Genetics*, vol. 31, no. 4, pp. 339-346. http://dx.doi.org/10.1038/ng0802-339. PMid:12149612.

YANG, Q.-F. and MENG, Q.-G., 2008. Discuss the animal model about inflammation. *Chinese Archives of Traditional Chinese Medicine*, vol. 26, no. 3, pp. 516-517.

ZHAO, M., YANG, N., YANG, B., JIANG, Y. and ZHANG, G., 2007. Structural characterization of water-soluble polysaccharides from Opuntia monacantha cladodes in relation to their anti-glycated activities. *Food Chemistry*, vol. 105, no. 4, pp. 1480-1486. http://dx.doi.org/10.1016/j.foodchem.2007.05.031.

ZHOU, J., LIANG, S., FANG, L., CHEN, L., TANG, M., XU, Y., FU, A., YANG, J. and WEI, Y., 2009. Quantitative proteomic analysis of HepG2 cells treated with quercetin suggests IQGAP1 involved in quercetin-induced regulation of cell proliferation and migration. *OMICS: A Journal of Integrative Biology*, vol. 13, no. 2, pp. 93-103. http://dx.doi.org/10.1089/omi.2008.0075. PMid:19207037.