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Autophagy mediated anticancer activity, antioxidant and antimicrobial potential of extracts from young twigs and leaves of *Juglansregia* growing in Kashmir Valley, India

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ABSTRACT
The aim of this research was to evaluate the phytochemical content, antimicrobial, antioxidant and anticancer effects of different solvent extracts of young twigs and leaves of Juglansregia. Methanolic, ethanolic and hexane extracts were evaluated for total phenolic and flavonoids content. DPPH scavenging, ABTS scavenging and FRAP assay was used to find out antioxidant potential of the extracts. Antimicrobial activity was checked by disc diffusion method and cell proliferation by MTT assay. Results showed high phenol and flavonoids content in all three extracts but high in ethanolic extract. Strong DPPH and ABTS scavenging and FRAP was reported for ethanolic extract while mild activity for methanolic and hexane extracts. All the three extracts showed mild to high antimicrobial activity against the tested bacterial strains. The MIC values obtained for ethanolic extract against Streptococcus mutans, Streptococcus salivarius, Streptococcus sanguis, and Staphylococcus aureus were significantly high than the other two extracts and comparable to that of reference control viz 0.29, 0.21, 0.45, and 0.19 µg/ml. Higher antiproliferative activity following a concentration-reliant pattern was revealed by all the extracts against the Hep-G2 cell line. Significantly high activity for ethanolic extract with IC_{50} value of 31.57 µg/ml in comparison to 33.93 µg/ml and 83.55 µg/ml for methanolic and hexane extracts. Therefore, mechanism studies were carried for ethanolic extract treated Hep-G2 cells indicating autophagy mediated antiproliferative effects. In conclusion, results showed autophagy mediated anticancer activity, antioxidant and antimicrobial potential of extracts from young twigs and leaves of Juglan sregia.
Keywords: *Juglan sregia, anticancer, autophagy, antioxidant, antimicrobial*

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INTRODUCTION

Medicinal and aromatic plants are a treasure of Mother Nature given as gift to human beings. These plants have high diversity of chemical compounds showing high value in each and every sector of living systems weather it is medicine or nutrition [1]. There are different classes of natural products present in medicinal plants with higher importance to pharmaceutical industry like alkaloids, flavonoids, terpenoids, coumarins, polyphenols, steroids etc. [2]. These phytochemicals have shown promising results in elimination of numerous human disorders to name a few asthma, anti-inflammatory, antioxidant, antibiotic, antiviral as well as anticancer [3,4]. The free radical species or reactive oxygen species or oxidative chemical species are the lethal chemical entities short lived but most dangerous to living systems [5]. These species can almost react everything that comes in their path. Thus the presence of these species in human and other living cells cause lethal damages to normal functions of a cell. The free radical species cause damage to cell genetic material, proteins and enzymes [6]. These species of chemicals are often associated with carcinogenesis and other harmful malignancies in humans [7]. Therefore, the antioxidants are wanted globally for trapping of such species to avoid lethal disorders. Natural products have been reported to possessing tremendous antioxidant potential especially phenols and flavonoids [8]. Several studies have reported in vitro and in vivo antioxidant potential of natural products. Due to their natural origin, natural products are considered as nontoxic and efficient antioxidants. The induction of natural antioxidants in daily diet could suppress the origination of hazardous health conditions in humans [9]. Natural products are the leading source of antiproliferative agents in cancer cells [10]. Several medicinal plant derived natural products have been found with antiproliferative effects against lethal cancer cells including cancer cells of lung, liver, gastric, colon, adenocarcinoma, leukemia etc. [11]. Natural products like taxol, paclitaxel, vinblastine and vincristine are now a part of approved chemotherapeutics [12]. Therefore, natural products have a tremendous potential

of inhibit cancer cell proliferation. Hepatocellular cancer is one of the harmful health conditions responsible for effecting huge human population and is ranked among top five lethal cancers globally [13]. Despite the availability of modern treatment methodologies for hepatocellular carcinoma, the overall survival is very low and disease relapse possess huge challenge to the researchers and scientists. The plant *Juglan sregia* belongs to the family of Juglandaceae and is an important medicinal plant [14]. This plant is found all over Kashmir Valley and bears strong ethnopharmacological importance both locally and internationally. The plant shows rich phytochemistry and pharmacological importance like anti-inflammatory, antimicrobial, antipyretic, hepatoprotective and anticancer [15]. Therefore, the current study was designed to investigate phytochemical content, antimicrobial, antioxidant and anti-liver cancer efficacy of three different solvent extracts of young twigs and leaves of *Juglansregia*.

MATERIAL AND METHODS

Plant material

The plant material of the *Juglansregia*, new born twigs and leaves, was collected from Tehsil: Chadoora, District: Budgam, Jammu and Kashmir, India, in first week of May, 2020. The Plant material was authenticated in the Department of Botany, Madhyanchal Professional University (MPU), Ratibad, Bhopal, Madhya Pradesh and a specimen was submitted their with reference number MPU-B-109.

Extract preparation

The plant material was collected and rinsed under tap water to eliminate any dirt. Then the twigs and roots were kept in dark for two weeks until they become dry. The dried plant material was crushed to powder in a grinder and stored in air tight plastic bags to avoid any contamination. The plant material was finally extracted with three different solvents that are methanol (70%), ethanol (70%) and hexane (80%) using refluxing in round bottom flask fitted with magnetic stirrer at 50°C for 72 h. The individual mixtures were then filtered followed by rotatory evaporation of the mixtures for moisture evaporation till the crude extract is obtained in each case. The experimental concentrations for each extract were prepared by dissolving in DMSO viz 15, 30, 60, 120, 240 and 480 µg/ml.

Quantitative analysis of phenols and flavonoids

The total phenolic content was measured by modified Folin–Ciocalteu method and total flavonoids content by the method of Ordonez et al. (2006) with slight modifications [16,17].

DPPH scavenging assay

The DPPH scavenging assay was taken as a measure to assess the antioxidant potential of different solvent extracts of new born twigs and leaves of *Juglansregia*. The DPPH solution was prepared in methanol to a concentration of 0.025 g/L. The 200 µL of DMSO solution were added to each well of 96-well plates followed by the addition of each extract (methanolic, ethanolic and hexane) concentrations viz 15, 30, 60, 120, 240 and 480 µg/ml. The ascorbic acid was used as reference control for each extract. This mixture was left to react in dark for 20 min at room temperature. Afterwards, each well plate was subjected to absorbance measurements using an ELISA microplate reader at 570 nm. The inhibition of DPPH radicals was expressed as percentage inhibition calculated using eq. 1. Where, A is the absorbance.

$$\%Inhibition = [A(control) - A(sample)/\ A(control)] - 100 \quad \text{.....eq.1}$$

FRAP assay

The ferric ion reduction by these three solvent extracts of *Juglan sregia* twigs and leaves was measured to major the antioxidant power. In brief, the FRAP solution was prepared by mixing 10 mMtripyridyltriazine, 300 mM sodium acetate and 20 mM ferric chloride in a ratio of 1:10:1 by volumes, respectively. The 3ml of this solution was individually added with different concentrations of the three extracts viz 15, 30, 60, 120, 240 and 480 µg/ml, and left for reacting for half an hour at 37°C. The ascorbic acid was used as reference control for each extract. This mixture was then subjected to absorbance studies using 590 nm of wavelength. The absorbance was directly a measure for ferric ion reducing power of the three extracts.

ABTS radical scavenging assay

The ABTS radical-scavenging activity of the three extracts was evaluated to determine their antioxidant potential. The free radical cations of ABTS were produced by reaction of 2.5 mM of potassium persulphate (5ml) to the ABTS stock solution (5ml) in absence of light for 24h. 5 µl of this solution was added with different concentrations of the plant extracts viz 15, 30, 60, 120, 240 and 480 µg/ml followed by incubation at room temperature for 10 min, separately. The ascorbic acid was used as reference control for each extract. Finally, the absorbance was detected at 730 nm and the percentage inhibition was calculated using eq.1.

Microbial culture

The microbes *Streptococcus mutans, Streptococcus salivarius, Streptococcus sanguis, and Staphylococcus aureus* wereprocured Microbial Type Culture Collection, Institute of Microbial Technology (IMTECH), Chandigarh, India. All the microbes were preserved in Muller-Hinton agar at 37°C till use.

Disc-diffusion method

The antimicrobial property of the different solvent extracts of new born leaves and twigs of *Juglan sregia* was evaluated using disc-diffusion method. Microbes were loaded to discs and spread using a sterile pipette tip. The discs were placed with 20 µL of each extract concentration and then relocated in Muller-Hinton agar. The tetracycline was used as reference control. All the inoculated plates bearing discs were incubated for 20 h at 37°C. Finally, the zone of inhibition of illustrating the antimicrobial activity was measured in millimeters. The minimum inhibition concentration (MIC) was considered as the minimum extract concentration at which no visible microbial growth is observed and it is expressed in µg/ml

Cell culture

The liver cancer cell line Hep-G2 cells were obtained from American Type Culture Collection (ATCC, Manassas, United States). The cells were cultured within DMEM for 24 h in moist conditions at 37°C within a 5% CO_2 incubator. The DMEM comprised of penicillin and streptomycin as antibiotics and 5% FBS.

MTT assay

In order to check the effects of extracts on cell proliferation of Hep-G2 cells, MTT assay was performed. In brief, the cultured cells with a concentration of 1×10^5 cells/well were loaded to 96-well plates followed by addition of different concentrations of methanolic, ethanolic and hexane extracts viz 15, 30, 60, 120, 240 and 480 µg/ml and incubated for 48 h. Post treatment, Hep-G2 cells were added with MTT reagent (20 µL) and further incubated for 4 h in absence of light. The MTT solution gets converted to formazan crystals in living cells. these crystals are dissolved in DMSO. Finally, absorbance of each sample was recorded using ELISA microplate reader set at 490 nm.

Transmission electron microscopy

To check the autophagy mediated antiproliferative effects of ethanolic extract against Hep-G2 cells, TEM analysis was used. Post treatment of Hep-G2 cells with different ethanolic extract concentrations (control, 60, 120 and 240 µg/ml) in 24-well plates, cells were washed in PBS and fixed in glutaraldehyde (2.5%). The washed cells then stained with the help of 2% osmium tetroxide and subsequently incubated for 2 h at room temperature. The treated and stained Hep-G2 cells were dehydrated with the help of ethanol and then immersed within pure acetone. Prior to the sectioning (5-7 µm) under Ultracut E ultra-thin slicer, the cells were placed in epoxy resin mixture. The ultrathin sections were stained using lead citrate and uranyl acetate (3%) followed by 20 min of incubation in dark at room temperature. Finally, the stained ultrathin sections were washed twice in distilled water and subsequently loaded to a transmission electron microscope (Philips Medical Systems., Eindhoven, Netherland)

Statistical analysis

Each experiment was replicated in three repetitions and the data were shown as mean ± SD. The statistical significant difference were determined using one way ANOVA and Tukey's test with the help of SPSS software version 15.0. The significant figure value was take as $*p<0.05$.

RESULTS AND DISCUSSION

Total phenols and flavonoids

In this study we, evaluated the phenolic and flavonoids content in different solvent extracts of new born twigs and leaves of *Juglansregia*. This plant has been previously shown of tremendous phytochemical composition with the presence of bioactive compounds like juglone and juglanin [18]. We herein found that the extracts of young twigs and leaves showed rich content of phenols and flavonoids with the higher amounts present in ethanolic extract 22 ± 1.13 and 10 ± 0.28 GAE/g of DPM (Table 1).

Antioxidant activity

The presence of high phenolic and flavonoids content in the extracts of young twigs and leaves of *Juglansregia* made us to search for their antioxidant potential. Previous studies have reported antioxidant activity for leaves, bark and roots of the plant but no such evidence was found in literature on young leaves and twigs of the plant [19]. We found that the three extracts: methanolic ethanolic and hexane extracts showed significant antioxidant activity against the radicals of DPPH, ferric ions and ABTS. The DPPH scavenging potential of the extracts was remarkable with enhancing pattern showing concentration-dependence. Ethanolic extract showed higher DPPH scavenging activity of 20% to 88% (15-480 µg/ml) than methanolic and hexane extracts respectively (Figure 1). The DPPH scavenging activity of ethanolic extract was comparable to that reference control ascorbic acid. The FRAP assay results revealed higher potential of the three extracts to reduce ferric ions in a concentration-reliant

fashion. Higher FRAP was witnessed for ethanolic extract followed by methanolic and hexane extract (Figure 2). The absorbance recorded for ethanolic extract was significant in comparison to that of ascorbic acid evidencing strong antioxidant potential of ethanolic extract. Similar results were recorded in case of ABTS radical scavenging assay, where ethanolic extracted revealed about 92% of ABTS radical inhibition at 480 µg/ml. These results were competent to that reference control ascorbic acid showing 97% inhibition of ABTS radicals (Figure 3).

Therefore, the above results show that ethanolic extract possess higher antioxidant activity than the methanolic and hexane extracts when compared to reference control. The higher antioxidant activity of the ethanolic extract was attributed to its higher phenolic and flavonoids content.

Table 1: Total Phenolic and Flavonoids content in the extracts from new born twigs and leaves of *Juglan sregia*.

S.No.	Quantitative analysis	Extracts from new born twigs and leaves of *Juglansregia*		
		Ethanolic	Methanolic	Hexane
1.	[a]Phenol	22 ± 1.13*	19± 1.22*	12± 1.17*
2.	[b]Flavonoids	10± 0.28*	9± 0.55*	7± 0.39*

a Representation in terms of mg of gallic acid equivalent (GAE)/g of DPM.
b Representation in terms of mg quercetin/g of DPM.
**p< 0.05*

Table 2: Antimicrobial activity of different solvent extracts of new born twigs and leaves of *Juglansregia* using disc-diffusion method

Microbes	Antimicrobial activity of different solvent extracts of new born twigs and leaves of *Juglansregia*							
	Methanolic		Ethanolic		Hexane		Tetracycline	
	Inhibition zone (mm)	MIC (mg/ml)	Inhibition zone (mm)	MIC (mg/ml)	Inhibition zone (mm)	MIC (mg/ml)	Inhibition zone (mm)	MIC (mg/ml)
S. mutans	17 ± 1.21	0.38	19 ± 0.93	0.29	8 ± 1.27	0.85	21 ± 0.84	0.02
S.salivarius	20 ± 1.13	0.30	25 ± 0.91	0.21	11 ± 1.15	0.78	28 ± 0.95	0.3
S. sanguis	9 ± 1.17	0.59	13 ± 1.19	0.45	5 ± 1.15	0.97	16 ± 0.84	0.05
S. aureus	23 ± 1.17	0.27	29 ± 1.12	0.19	19 ± 1.23	0.63	33 ± 1.10	0.02

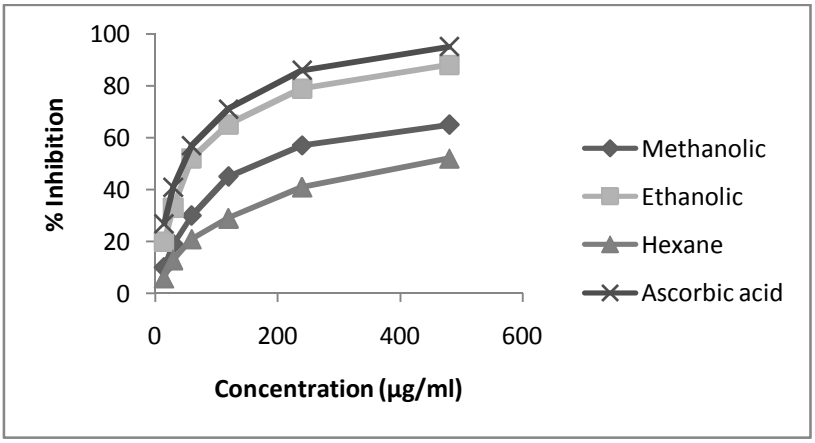

Figure 1: Relative DPPH scavenging activity of methanolic, ethanolic and hexane extracts of young twigs and leaves of *Juglansregia* against the reference control ascorbic acid. Each experiment was replicated in triplicate replicas.

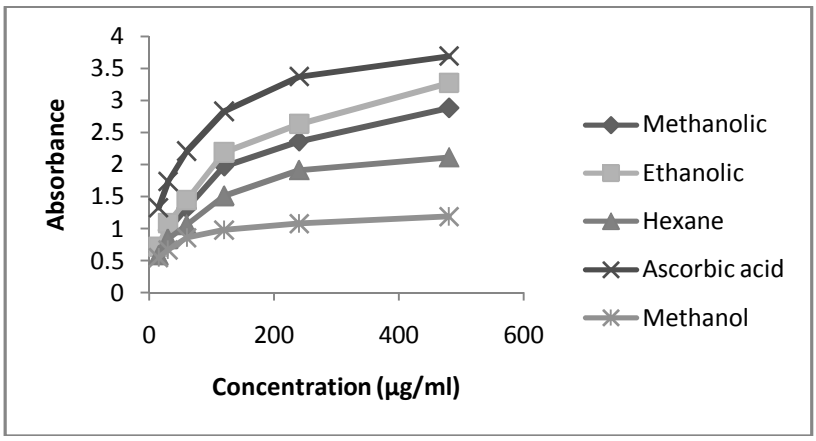

Figure 2: Relative ferric ion reducing activity of methanolic, ethanolic and hexane extracts of young twigs and leaves of *Juglan sregia* against the reference control ascorbic acid. Each experiment was replicated in triplicate replicas.

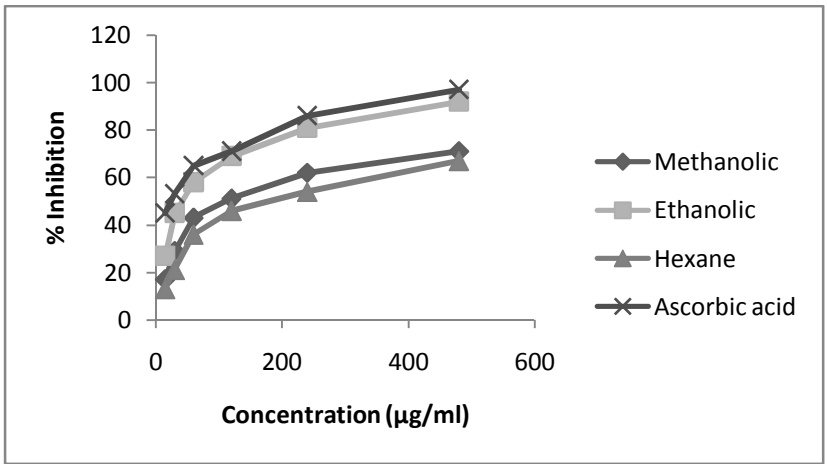

Figure 3: Relative ABTS radical scavenging activity of methanolic, ethanolic and hexane extracts of young twigs and leaves of *Juglan sregia* against the reference control ascorbic acid. Each experiment was replicated in triplicate replicas.

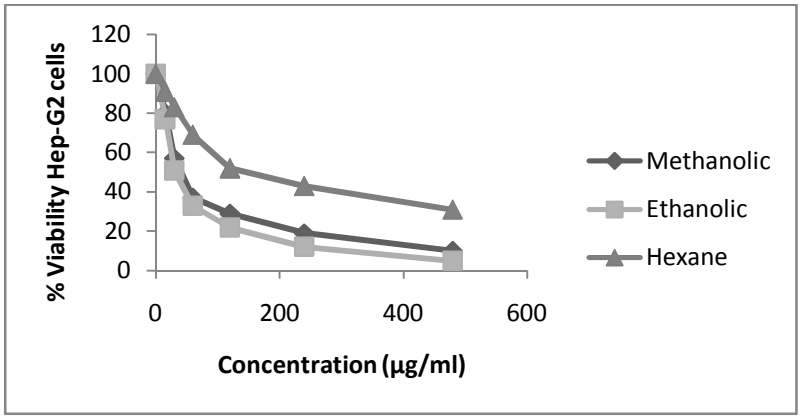

Figure 4: The MTT assay was executed to check the antiproliferative effects of methanolic, ethanolic and hexane extracts of young twigs and leaves of *Juglan sregia* against Hep-G2 cells. Results suggested dose-reliant inhibition of the proliferation by all the three extracts but highest for ethanolic extract. Each experiment was replicated in triplicate replicas.

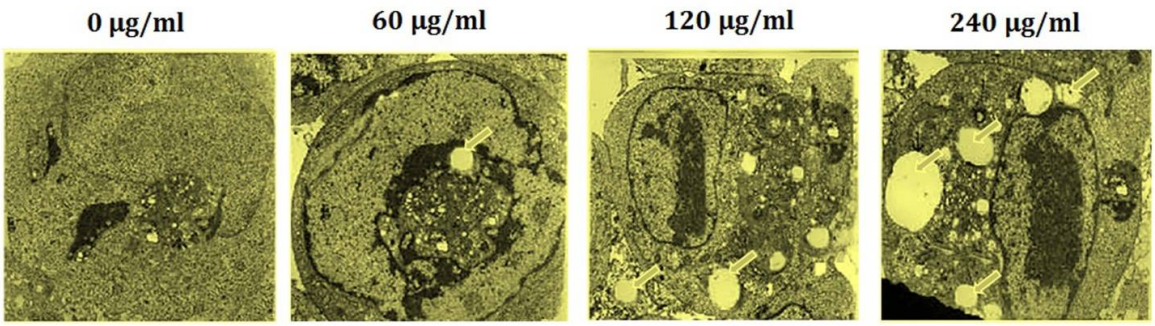

Figure 5:TEM analysis of ethanolic extract treated Hep-G2 cells. The figure represents the formation of autophagosomes in treated cells compared to controls as indicated by pointed arrows. Each experiment was replicated in triplicate replicas.

Antimicrobial activity

Medicinal plants show strong antimicrobial activity against bacteria, fungi, and viruses. Plants synthesize secondary metabolites as protection against any kind of threat including microbial infections. Therefore, numerous plant derived secondary metabolites on isolation reveal strong antibiotic potential and some of the natural products are serving modern day pharmaceutical industry assisting as drugs against microbial infections to humans. The plant *Juglansregia*has been previously reported of antimicrobial activities against different bacterial and microbial strains [20]. Our results were similar to the previous studies reporting antimicrobial activity but here we used first time new born leaves and twigs.

Antimicrobial activity of the methanolic, ethanolic and hexane extracts of young leaves and twigs of *Juglan sregia* was determined using disc-diffusion method. The microbes tested in this study include *Streptococcus mutans, Streptococcus salivarius, Streptococcus sanguis, and Staphylococcus aureus*. Each extract showed different potential of microbial inhibition depending on microbe we are testing. It was observed that all the three extracts showed remarkable inhibition against selected bacterial strains. The zone of inhibition (mm) for ethanolic extract yielded a healthy score of 19 ± 0.93, 25 ± 0.91, 13 ± 1.19 and 29 ± 1.12 against the reference control 21 ± 0.84, 28 ± 0.95, 16 ± 0.84 and 33 ± 1.10, respectively. The MIC values were efficient for all the tested extracts while ethanolic extract showed strong MIC value of 0.29, 0.21, 0.45 and 0.19 µg/ml, respectively, in comparison to the reference control. Strong antimicrobial activity was reported for *Streptococcus sanguis, and Staphylococcus aureus* (Table 2).

Antiproliferative activity

Previous studies have reported anticancer effects of *Juglan sregia* against the breast and colon cancer cells [21]. Herein, we estimated the antiproliferative effects of the three extracts of young leaves and twigs of *Juglan sregia*. The results showed remarkable antiproliferative effects for the three extracts with against malignant liver cancer cells Hep-G2 cells in a concentration-dependence pattern. The higher antiproliferative activity was recorded for ethanolic extract with an IC_{50} value of 31.57 µg/ml in comparison to 33.93 µg/ml and 83.55 µg/ml for methanol and hexane extracts (Figure 4). Therefore, higher phytochemical content of ethanolic extract was considered the reason for higher antiproliferative activity against Hep-G2 cells.

Autophagy activation

Autophagy is one of the targets of potential chemotherapeutic drugs in cancer cells [22]. It is a type II programmed cell death stimulated under stress, starvation and disintegration of accumulated macro proteins [23]. Therefore, apoptosis plays vital role in maintaining regular homeostasis, functioning and health of cells and tissues. Autophagy stimulation in cancer cells prevents cancer cells from growth and differentiation. Autophagy is a complex process activated by different genes including LC3B-I and LC3B-II [22]. Several natural products induce their anticancer effects via stimulation of autophagy. Juglone and Juglanin from the plant *Juglansregia* have been reported to induce autophagy mediated anticancer effects[18].

Herein, in this research we that ethanolic extract showed significant antiproliferative effects against liver cancer Hep-G2 cells. Therefore, mechanism search for its autophagy mediation was evaluated using TEM analysis. The results clearly suggest formation of autophagosomes in extract treated cells than untreated control cells (Figure 5). Autophagosomes are a complete hallmark for autophagy. Therefore, the results revealed that ethanolic extract induced antiproliferative effects in Hep-G2 cells via stimulation of autophagy.

CONCLUSION

In conclusion, the results of this research investigation suggested that the different solvent extracts from young twigs and leaves of *Juglan sregia* induce antimicrobial, antioxidant and anticancer effects mediated via autophagy induction. Therefore, our study could be a leading platform for evaluation of anticancer bioactive constituents of young leaves and twigs of *Juglan sregia*.

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CONFLICT OF INTEREST

None to declare.

AUTHOR CONTRIBUTION

All the authors contributed equally in design, execution, writing and review of this research.

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Autophagy mediated anticancer activity, antioxidant and antimicrobial potential of extracts from young twigs and leaves of *Juglansregia* growing in Kashmir Valley, India

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ABSTRACT
The aim of this research was to evaluate the phytochemical content, antimicrobial, antioxidant and anticancer effects of different solvent extracts of young twigs and leaves of Juglansregia. Methanolic, ethanolic and hexane extracts were evaluated for total phenolic and flavonoids content. DPPH scavenging, ABTS scavenging and FRAP assay was used to find out antioxidant potential of the extracts. Antimicrobial activity was checked by disc diffusion method and cell proliferation by MTT assay. Results showed high phenol and flavonoids content in all three extracts but high in ethanolic extract. Strong DPPH and ABTS scavenging and FRAP was reported for ethanolic extract while mild activity for methanolic and hexane extracts. All the three extracts showed mild to high antimicrobial activity against the tested bacterial strains. The MIC values obtained for ethanolic extract against Streptococcus mutans, Streptococcus salivarius, Streptococcus sanguis, and Staphylococcus aureus were significantly high than the other two extracts and comparable to that of reference control viz 0.29, 0.21, 0.45, and 0.19 µg/ml. Higher antiproliferative activity following a concentration-reliant pattern was revealed by all the extracts against the Hep-G2 cell line. Significantly high activity for ethanolic extract with IC_{50} value of 31.57 µg/ml in comparison to 33.93 µg/ml and 83.55 µg/ml for methanolic and hexane extracts. Therefore, mechanism studies were carried for ethanolic extract treated Hep-G2 cells indicating autophagy mediated antiproliferative effects. In conclusion, results showed autophagy mediated anticancer activity, antioxidant and antimicrobial potential of extracts from young twigs and leaves of Juglan sregia.
Keywords: *Juglan sregia, anticancer, autophagy, antioxidant, antimicrobial*

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INTRODUCTION

Medicinal and aromatic plants are a treasure of Mother Nature given as gift to human beings. These plants have high diversity of chemical compounds showing high value in each and every sector of living systems weather it is medicine or nutrition [1]. There are different classes of natural products present in medicinal plants with higher importance to pharmaceutical industry like alkaloids, flavonoids, terpenoids, coumarins, polyphenols, steroids etc. [2]. These phytochemicals have shown promising results in elimination of numerous human disorders to name a few asthma, anti-inflammatory, antioxidant, antibiotic, antiviral as well as anticancer [3,4]. The free radical species or reactive oxygen species or oxidative chemical species are the lethal chemical entities short lived but most dangerous to living systems [5]. These species can almost react everything that comes in their path. Thus the presence of these species in human and other living cells cause lethal damages to normal functions of a cell. The free radical species cause damage to cell genetic material, proteins and enzymes [6]. These species of chemicals are often associated with carcinogenesis and other harmful malignancies in humans [7]. Therefore, the antioxidants are wanted globally for trapping of such species to avoid lethal disorders. Natural products have been reported to possessing tremendous antioxidant potential especially phenols and flavonoids [8]. Several studies have reported in vitro and in vivo antioxidant potential of natural products. Due to their natural origin, natural products are considered as nontoxic and efficient antioxidants. The induction of natural antioxidants in daily diet could suppress the origination of hazardous health conditions in humans [9]. Natural products are the leading source of antiproliferative agents in cancer cells [10]. Several medicinal plant derived natural products have been found with antiproliferative effects against lethal cancer cells including cancer cells of lung, liver, gastric, colon, adenocarcinoma, leukemia etc. [11]. Natural products like taxol, paclitaxel, vinblastine and vincristine are now a part of approved chemotherapeutics [12]. Therefore, natural products have a tremendous potential

of inhibit cancer cell proliferation. Hepatocellular cancer is one of the harmful health conditions responsible for effecting huge human population and is ranked among top five lethal cancers globally [13]. Despite the availability of modern treatment methodologies for hepatocellular carcinoma, the overall survival is very low and disease relapse possess huge challenge to the researchers and scientists. The plant *Juglan sregia* belongs to the family of Juglandaceae and is an important medicinal plant [14]. This plant is found all over Kashmir Valley and bears strong ethnopharmacological importance both locally and internationally. The plant shows rich phytochemistry and pharmacological importance like anti-inflammatory, antimicrobial, antipyretic, hepatoprotective and anticancer [15]. Therefore, the current study was designed to investigate phytochemical content, antimicrobial, antioxidant and anti-liver cancer efficacy of three different solvent extracts of young twigs and leaves of *Juglansregia*.

MATERIAL AND METHODS

Plant material

The plant material of the *Juglansregia*, new born twigs and leaves, was collected from Tehsil: Chadoora, District: Budgam, Jammu and Kashmir, India, in first week of May, 2020. The Plant material was authenticated in the Department of Botany, Madhyanchal Professional University (MPU), Ratibad, Bhopal, Madhya Pradesh and a specimen was submitted their with reference number MPU-B-109.

Extract preparation

The plant material was collected and rinsed under tap water to eliminate any dirt. Then the twigs and roots were kept in dark for two weeks until they become dry. The dried plant material was crushed to powder in a grinder and stored in air tight plastic bags to avoid any contamination. The plant material was finally extracted with three different solvents that are methanol (70%), ethanol (70%) and hexane (80%) using refluxing in round bottom flask fitted with magnetic stirrer at 50°C for 72 h. The individual mixtures were then filtered followed by rotatory evaporation of the mixtures for moisture evaporation till the crude extract is obtained in each case. The experimental concentrations for each extract were prepared by dissolving in DMSO viz 15, 30, 60, 120, 240 and 480 µg/ml.

Quantitative analysis of phenols and flavonoids

The total phenolic content was measured by modified Folin–Ciocalteu method and total flavonoids content by the method of Ordonez et al. (2006) with slight modifications [16,17].

DPPH scavenging assay

The DPPH scavenging assay was taken as a measure to assess the antioxidant potential of different solvent extracts of new born twigs and leaves of *Juglansregia*. The DPPH solution was prepared in methanol to a concentration of 0.025 g/L. The 200 µL of DMSO solution were added to each well of 96-well plates followed by the addition of each extract (methanolic, ethanolic and hexane) concentrations viz 15, 30, 60, 120, 240 and 480 µg/ml. The ascorbic acid was used as reference control for each extract. This mixture was left to react in dark for 20 min at room temperature. Afterwards, each well plate was subjected to absorbance measurements using an ELISA microplate reader at 570 nm. The inhibition of DPPH radicals was expressed as percentage inhibition calculated using eq. 1. Where, A is the absorbance.

$$\%Inhibition = [A(control) - A(sample)/\ A(control)] - 100 \quad \text{.....eq.1}$$

FRAP assay

The ferric ion reduction by these three solvent extracts of *Juglan sregia* twigs and leaves was measured to major the antioxidant power. In brief, the FRAP solution was prepared by mixing 10 mMtripyridyltriazine, 300 mM sodium acetate and 20 mM ferric chloride in a ratio of 1:10:1 by volumes, respectively. The 3ml of this solution was individually added with different concentrations of the three extracts viz 15, 30, 60, 120, 240 and 480 µg/ml, and left for reacting for half an hour at 37°C. The ascorbic acid was used as reference control for each extract. This mixture was then subjected to absorbance studies using 590 nm of wavelength. The absorbance was directly a measure for ferric ion reducing power of the three extracts.

ABTS radical scavenging assay

The ABTS radical-scavenging activity of the three extracts was evaluated to determine their antioxidant potential. The free radical cations of ABTS were produced by reaction of 2.5 mM of potassium persulphate (5ml) to the ABTS stock solution (5ml) in absence of light for 24h. 5 µl of this solution was added with different concentrations of the plant extracts viz 15, 30, 60, 120, 240 and 480 µg/ml followed by incubation at room temperature for 10 min, separately. The ascorbic acid was used as reference control for each extract. Finally, the absorbance was detected at 730 nm and the percentage inhibition was calculated using eq.1.

Microbial culture

The microbes *Streptococcus mutans, Streptococcus salivarius, Streptococcus sanguis, and Staphylococcus aureus* wereprocured Microbial Type Culture Collection, Institute of Microbial Technology (IMTECH), Chandigarh, India. All the microbes were preserved in Muller-Hinton agar at 37°C till use.

Disc-diffusion method

The antimicrobial property of the different solvent extracts of new born leaves and twigs of *Juglan sregia* was evaluated using disc-diffusion method. Microbes were loaded to discs and spread using a sterile pipette tip. The discs were placed with 20 µL of each extract concentration and then relocated in Muller-Hinton agar. The tetracycline was used as reference control. All the inoculated plates bearing discs were incubated for 20 h at 37°C. Finally, the zone of inhibition of illustrating the antimicrobial activity was measured in millimeters. The minimum inhibition concentration (MIC) was considered as the minimum extract concentration at which no visible microbial growth is observed and it is expressed in µg/ml

Cell culture

The liver cancer cell line Hep-G2 cells were obtained from American Type Culture Collection (ATCC, Manassas, United States). The cells were cultured within DMEM for 24 h in moist conditions at 37°C within a 5% CO_2 incubator. The DMEM comprised of penicillin and streptomycin as antibiotics and 5% FBS.

MTT assay

In order to check the effects of extracts on cell proliferation of Hep-G2 cells, MTT assay was performed. In brief, the cultured cells with a concentration of 1×10^5 cells/well were loaded to 96-well plates followed by addition of different concentrations of methanolic, ethanolic and hexane extracts viz 15, 30, 60, 120, 240 and 480 µg/ml and incubated for 48 h. Post treatment, Hep-G2 cells were added with MTT reagent (20 µL) and further incubated for 4 h in absence of light. The MTT solution gets converted to formazan crystals in living cells. these crystals are dissolved in DMSO. Finally, absorbance of each sample was recorded using ELISA microplate reader set at 490 nm.

Transmission electron microscopy

To check the autophagy mediated antiproliferative effects of ethanolic extract against Hep-G2 cells, TEM analysis was used. Post treatment of Hep-G2 cells with different ethanolic extract concentrations (control, 60, 120 and 240 µg/ml) in 24-well plates, cells were washed in PBS and fixed in glutaraldehyde (2.5%). The washed cells then stained with the help of 2% osmium tetroxide and subsequently incubated for 2 h at room temperature. The treated and stained Hep-G2 cells were dehydrated with the help of ethanol and then immersed within pure acetone. Prior to the sectioning (5-7 µm) under Ultracut E ultra-thin slicer, the cells were placed in epoxy resin mixture. The ultrathin sections were stained using lead citrate and uranyl acetate (3%) followed by 20 min of incubation in dark at room temperature. Finally, the stained ultrathin sections were washed twice in distilled water and subsequently loaded to a transmission electron microscope (Philips Medical Systems., Eindhoven, Netherland)

Statistical analysis

Each experiment was replicated in three repetitions and the data were shown as mean ± SD. The statistical significant difference were determined using one way ANOVA and Tukey's test with the help of SPSS software version 15.0. The significant figure value was take as $*p<0.05$.

RESULTS AND DISCUSSION

Total phenols and flavonoids

In this study we, evaluated the phenolic and flavonoids content in different solvent extracts of new born twigs and leaves of *Juglansregia*. This plant has been previously shown of tremendous phytochemical composition with the presence of bioactive compounds like juglone and juglanin [18]. We herein found that the extracts of young twigs and leaves showed rich content of phenols and flavonoids with the higher amounts present in ethanolic extract 22 ± 1.13 and 10 ± 0.28 GAE/g of DPM (Table 1).

Antioxidant activity

The presence of high phenolic and flavonoids content in the extracts of young twigs and leaves of *Juglansregia* made us to search for their antioxidant potential. Previous studies have reported antioxidant activity for leaves, bark and roots of the plant but no such evidence was found in literature on young leaves and twigs of the plant [19]. We found that the three extracts: methanolic ethanolic and hexane extracts showed significant antioxidant activity against the radicals of DPPH, ferric ions and ABTS. The DPPH scavenging potential of the extracts was remarkable with enhancing pattern showing concentration-dependence. Ethanolic extract showed higher DPPH scavenging activity of 20% to 88% (15-480 µg/ml) than methanolic and hexane extracts respectively (Figure 1). The DPPH scavenging activity of ethanolic extract was comparable to that reference control ascorbic acid. The FRAP assay results revealed higher potential of the three extracts to reduce ferric ions in a concentration-reliant

fashion. Higher FRAP was witnessed for ethanolic extract followed by methanolic and hexane extract (Figure 2). The absorbance recorded for ethanolic extract was significant in comparison to that of ascorbic acid evidencing strong antioxidant potential of ethanolic extract. Similar results were recorded in case of ABTS radical scavenging assay, where ethanolic extracted revealed about 92% of ABTS radical inhibition at 480 µg/ml. These results were competent to that reference control ascorbic acid showing 97% inhibition of ABTS radicals (Figure 3).

Therefore, the above results show that ethanolic extract possess higher antioxidant activity than the methanolic and hexane extracts when compared to reference control. The higher antioxidant activity of the ethanolic extract was attributed to its higher phenolic and flavonoids content.

Table 1: Total Phenolic and Flavonoids content in the extracts from new born twigs and leaves of *Juglan sregia*.

S.No.	Quantitative analysis	Extracts from new born twigs and leaves of *Juglansregia*		
		Ethanolic	Methanolic	Hexane
1.	[a]Phenol	22 ± 1.13*	19± 1.22*	12± 1.17*
2.	[b]Flavonoids	10± 0.28*	9± 0.55*	7± 0.39*

a Representation in terms of mg of gallic acid equivalent (GAE)/g of DPM.
b Representation in terms of mg quercetin/g of DPM.
**p< 0.05*

Table 2: Antimicrobial activity of different solvent extracts of new born twigs and leaves of *Juglansregia* using disc-diffusion method

Microbes	Antimicrobial activity of different solvent extracts of new born twigs and leaves of *Juglansregia*							
	Methanolic		Ethanolic		Hexane		Tetracycline	
	Inhibition zone (mm)	MIC (mg/ml)	Inhibition zone (mm)	MIC (mg/ml)	Inhibition zone (mm)	MIC (mg/ml)	Inhibition zone (mm)	MIC (mg/ml)
S. mutans	17 ± 1.21	0.38	19 ± 0.93	0.29	8 ± 1.27	0.85	21 ± 0.84	0.02
S.salivarius	20 ± 1.13	0.30	25 ± 0.91	0.21	11 ± 1.15	0.78	28 ± 0.95	0.3
S. sanguis	9 ± 1.17	0.59	13 ± 1.19	0.45	5 ± 1.15	0.97	16 ± 0.84	0.05
S. aureus	23 ± 1.17	0.27	29 ± 1.12	0.19	19 ± 1.23	0.63	33 ± 1.10	0.02

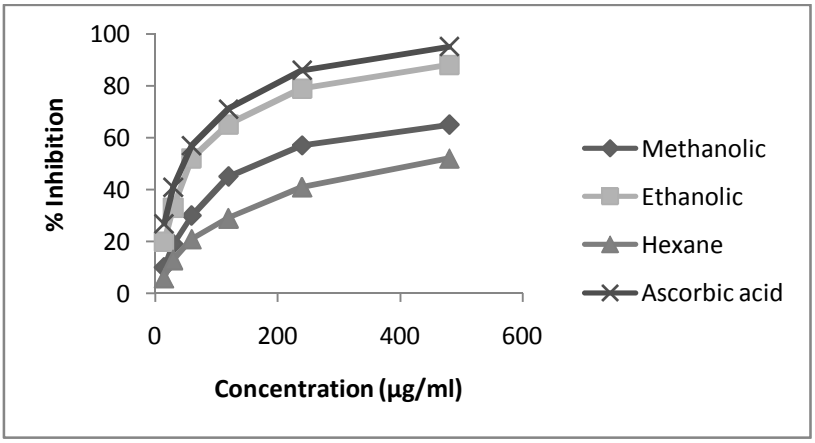

Figure 1: Relative DPPH scavenging activity of methanolic, ethanolic and hexane extracts of young twigs and leaves of *Juglansregia* against the reference control ascorbic acid. Each experiment was replicated in triplicate replicas.

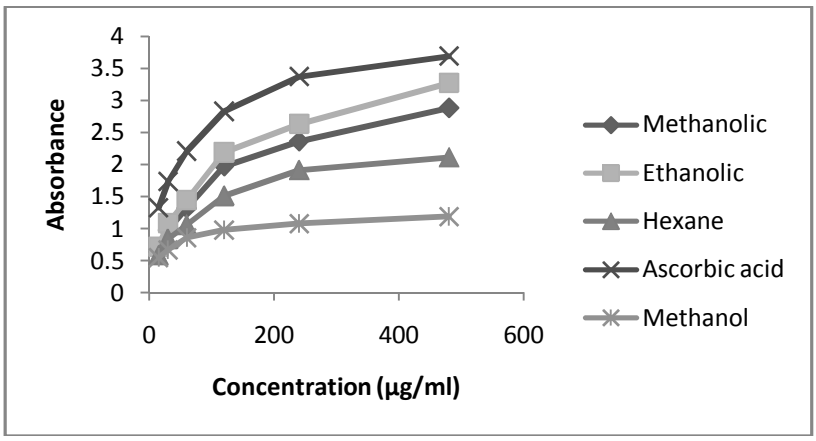

Figure 2: Relative ferric ion reducing activity of methanolic, ethanolic and hexane extracts of young twigs and leaves of *Juglan sregia* against the reference control ascorbic acid. Each experiment was replicated in triplicate replicas.

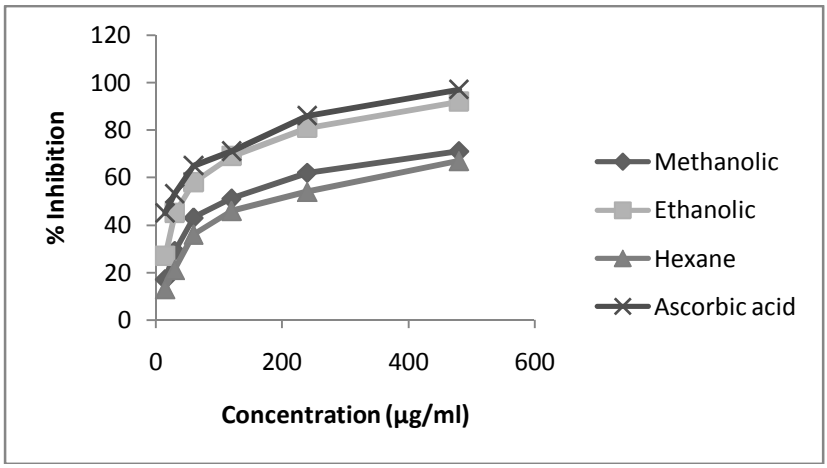

Figure 3: Relative ABTS radical scavenging activity of methanolic, ethanolic and hexane extracts of young twigs and leaves of *Juglan sregia* against the reference control ascorbic acid. Each experiment was replicated in triplicate replicas.

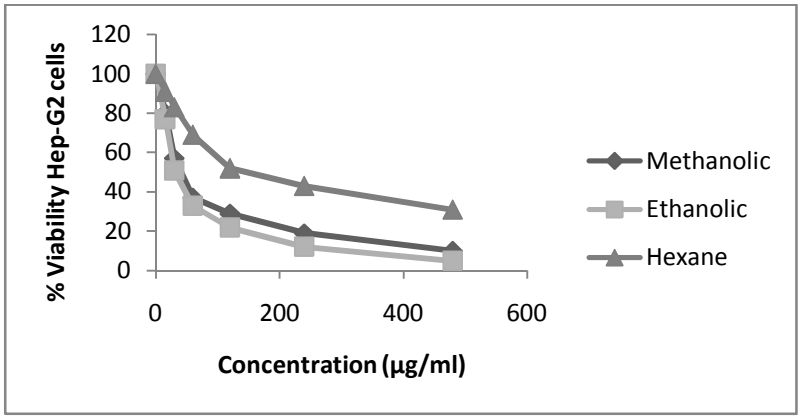

Figure 4: The MTT assay was executed to check the antiproliferative effects of methanolic, ethanolic and hexane extracts of young twigs and leaves of *Juglan sregia* against Hep-G2 cells. Results suggested dose-reliant inhibition of the proliferation by all the three extracts but highest for ethanolic extract. Each experiment was replicated in triplicate replicas.

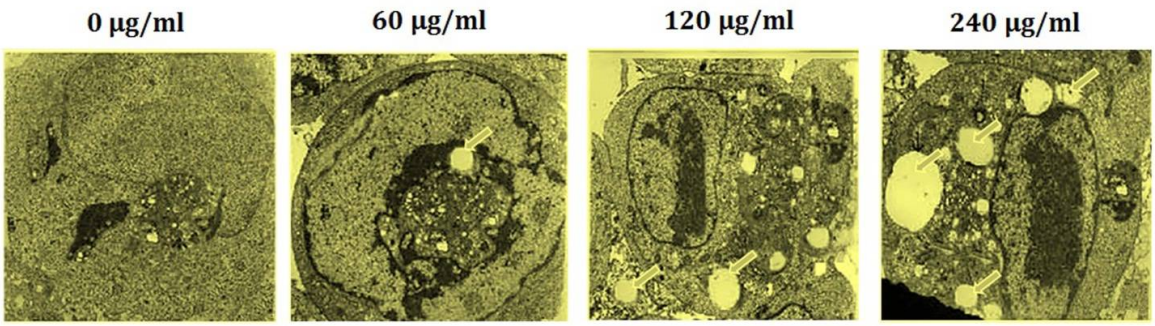

Figure 5:TEM analysis of ethanolic extract treated Hep-G2 cells. The figure represents the formation of autophagosomes in treated cells compared to controls as indicated by pointed arrows. Each experiment was replicated in triplicate replicas.

Antimicrobial activity

Medicinal plants show strong antimicrobial activity against bacteria, fungi, and viruses. Plants synthesize secondary metabolites as protection against any kind of threat including microbial infections. Therefore, numerous plant derived secondary metabolites on isolation reveal strong antibiotic potential and some of the natural products are serving modern day pharmaceutical industry assisting as drugs against microbial infections to humans. The plant *Juglansregia*has been previously reported of antimicrobial activities against different bacterial and microbial strains [20]. Our results were similar to the previous studies reporting antimicrobial activity but here we used first time new born leaves and twigs.

Antimicrobial activity of the methanolic, ethanolic and hexane extracts of young leaves and twigs of *Juglan sregia* was determined using disc-diffusion method. The microbes tested in this study include *Streptococcus mutans, Streptococcus salivarius, Streptococcus sanguis, and Staphylococcus aureus*. Each extract showed different potential of microbial inhibition depending on microbe we are testing. It was observed that all the three extracts showed remarkable inhibition against selected bacterial strains. The zone of inhibition (mm) for ethanolic extract yielded a healthy score of 19 ± 0.93, 25 ± 0.91, 13 ± 1.19 and 29 ± 1.12 against the reference control 21 ± 0.84, 28 ± 0.95, 16 ± 0.84 and 33 ± 1.10, respectively. The MIC values were efficient for all the tested extracts while ethanolic extract showed strong MIC value of 0.29, 0.21, 0.45 and 0.19 µg/ml, respectively, in comparison to the reference control. Strong antimicrobial activity was reported for *Streptococcus sanguis, and Staphylococcus aureus* (Table 2).

Antiproliferative activity

Previous studies have reported anticancer effects of *Juglan sregia* against the breast and colon cancer cells [21]. Herein, we estimated the antiproliferative effects of the three extracts of young leaves and twigs of *Juglan sregia*. The results showed remarkable antiproliferative effects for the three extracts with against malignant liver cancer cells Hep-G2 cells in a concentration-dependence pattern. The higher antiproliferative activity was recorded for ethanolic extract with an IC_{50} value of 31.57 µg/ml in comparison to 33.93 µg/ml and 83.55 µg/ml for methanol and hexane extracts (Figure 4). Therefore, higher phytochemical content of ethanolic extract was considered the reason for higher antiproliferative activity against Hep-G2 cells.

Autophagy activation

Autophagy is one of the targets of potential chemotherapeutic drugs in cancer cells [22]. It is a type II programmed cell death stimulated under stress, starvation and disintegration of accumulated macro proteins [23]. Therefore, apoptosis plays vital role in maintaining regular homeostasis, functioning and health of cells and tissues. Autophagy stimulation in cancer cells prevents cancer cells from growth and differentiation. Autophagy is a complex process activated by different genes including LC3B-I and LC3B-II [22]. Several natural products induce their anticancer effects via stimulation of autophagy. Juglone and Juglanin from the plant *Juglansregia* have been reported to induce autophagy mediated anticancer effects[18].

Herein, in this research we that ethanolic extract showed significant antiproliferative effects against liver cancer Hep-G2 cells. Therefore, mechanism search for its autophagy mediation was evaluated using TEM analysis. The results clearly suggest formation of autophagosomes in extract treated cells than untreated control cells (Figure 5). Autophagosomes are a complete hallmark for autophagy. Therefore, the results revealed that ethanolic extract induced antiproliferative effects in Hep-G2 cells via stimulation of autophagy.

CONCLUSION

In conclusion, the results of this research investigation suggested that the different solvent extracts from young twigs and leaves of *Juglan sregia* induce antimicrobial, antioxidant and anticancer effects mediated via autophagy induction. Therefore, our study could be a leading platform for evaluation of anticancer bioactive constituents of young leaves and twigs of *Juglan sregia*.

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CONFLICT OF INTEREST

None to declare.

AUTHOR CONTRIBUTION

All the authors contributed equally in design, execution, writing and review of this research.

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