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In vitro anticancer and antioxidant potential of *Cestrum* species

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Abstract: The methanolic extract of leaves and stem of *Cestrum nocturnum* and *Cestrum diurnum* were investigated for their antioxidant and anticancer attribute through standard methods. 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay was carried out to estimate the antioxidant activity of the extracts. Whereas, anticancer potential of extracts were tested against colon cancer cell line, HCT 116 and acute myeloid leukemia (AML) cell lines, THP-1. Results showed that extracts of both plants exhibited a very strong antioxidant activity in a dose dependent manner. In addition, both extracts efficiently increased the cell death in two different cancer cell lines. Moreover, DNA fragmentation analysis further strengthens the anticancer potential of extracts of both types of plants. Current study, therefore, provide a preliminary data highlighting the antioxidant and anticancer activities of methanolic extract of leaves and stem of *Cestrum nocturnum* and *Cestrum diurnum*.

Keywords: Antioxidant activity, DPPH assay, anti-cancer activity, *Cestrum* spp., colon cell lines.

INTRODUCTION

Cancer is one of the leading causes of death and rapidly becoming global pandemic (Jemal *et al*.2009). It is still a major challenge being faced by both developed and developing countries. Although the methods of cancer diagnosis and therapy have made a rapid progress recently, the efficacy of cancer treatments has not been improved significantly yet. Currently, the prevailing approaches to treat malignant tumors include surgery, radiation, immunomodulating and chemotherapy. However, most of the chemotherapeutic drugs present plenty of side effects. Therefore, searching for safe and effective antitumor agents is the aim of current anticancer research (Dai and Mumper, 2010). Many natural products discovered from medicinal plants, or secondary metabolites such as terpenoids, phenolic acids, lignans, tannins, flavonoids, quinones, coumarins, alkaloids, which exhibit significant antioxidant and other activities, have played an important role in treatment of cancer (Kaur *et al*. 2011; Khan *et al*. 2018).

Studies have shown that many of antioxidant compounds possess anti-inflammatory, antitumor, anti-mutagenic and anti-carcinogenic activities (Sala *et al.* 2002). Medicinal plants have therefore; become a focal point to improve the present and future health needs against cancer. Secondary metabolites of medicinal plants maintain normal health and cure various diseases including cancer with less harmful effects (Harun-ur-Fiashid *et al.* 2002; Khan, 2017).

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The genus *Cestrum* from family solanaceae comprises of more than 300 species which are distributed in many tropical and subtropical regions throughout the world including India, southern China, Australia, USA, Bangladesh and Pakistan (Al-Reza *et al.* 2009; 2010). Among which, *C. nocturnum* and *C. diurnum* are commonly known plants and are pronounced in Asia as raat ki rani (queen of the night) and din ka raja (king of the day), respectively. *C. nocturnum* possesses many pharmacological properties such as anti-inflammatory, analgesic (Mazumder *et al.* 2010), antimicrobial (Khan *et al.* 2011), anti-epileptic (Perez-Saad and Buznego, 2008), anti-cancer (Zhong *et al.* 2008) and insecticidal (Savchenko *et al.* 2000) activities. Plant is also being used as a local anesthetic and CNS depressant agent (Zeng *et al.* 2002). Important phyto-constituents including many flavonoids, alkaloids and phenols have been reported in *C. nocturnum* (Prasad *et al.* 2013). Most of these flavonoids have hepatoprotective activity (Ali et *al.* 2013).

Medicinal properties of *C. diurnum* are also well documented in several literatures whereas, in some cases, toxicity to humans and livestock has been reported. The leaves contain a carcinogenic glycoside called 1, 25-dihydroxychlorocalciferol that leads to vitamin D toxicity with elevated serum Ca^{2+} and deposition of calcium in soft tissues (Mello, 2003). Comparative anticancer and antioxidant activities of the stem and leaves of these plants have not been scientifically studied. Therefore, aim of the present work was to compare and report the anticancer and antioxidant activity of these plants.

MATERIALS AND METHODS

Plant collection and extract preparation

C. nocturnum and *C. diurnum* were collected from PCSIR Labs. Complex, Lahore, Pakistan and their herbaria were authenticated by Prof. Dr. A.N. Khalid (Herbarium, Department of Botany, University of Punjab, Lahore, Pakistan). Voucher specimens (BDSS # 1601, BDSS # 1602) were also deposited in the same herbarium.

Leaves and stems of both plants were shade dried at room temperature for 3 days followed by coarsely grinding. 200g of powdered plant material was extracted with methanol (500 mL×3) overnight (cold maceration) and filtered. Filtrate was completely evaporated under reduced pressure using rotary evaporator. Finally, extracts of *C. nocturnum* leaves (CNL), *C. nocturnum* stem (CNS), *C. diurnum* leaves (CDL) and *C. diurnum* stem (CDS) were obtained. Preliminary phytochemical study of extracts was carried out by standard methods.

Cell culture

HCT-116 human colon cancer (ATCC® CCL247™) and THP-1 acute myeloid leukemia (AML) cell lines (ATCC® TIB202™) were purchased from the American Type Culture Collection (ATCC). Cell Cultures were maintained at 37^{o}C in a humidified incubator with 5% CO_2 in RPMI 1640 media (ATCC, 30-2001), supplemented with 10% fetus bovine serum (ATCC, 30-2021) and penicillin–streptomycin solution (ATCC, 30-2300).

MTT Assay

90% confluent HCT-116 and THP-1 cells were harvested, counted with haemocytometer and seeded. For harvesting, HCT-116 (adherent cell types) cells were trypsinized with 1ml trypsin-EDTA solution (0.25% w/v trypsin and 0.53 mM EDTA). After 8-10 minutes of incubation, 2mL culture media was added to stop trypsin activity. While, THP-1 cells (suspending cells) were harvested and counted without trypsinization. 1×10^6 cells/mL of both types of cells was seeded in sterile 96-well plates separately. Cells were exposed to different concentrations of plant extracts (25, 50, 75, 100 and 200µg/mL) immediately forTHP-1 cells and after 24 hr for adherent cell lineHCT-116 cells. Cells were further incubated for a period of 24 and 48 hrs. Cells without plant extracts were taken as negative control whereas, cells treated with Triton X-100 was kept as positive control. Wells with media and without cells or extracts were taken as blank. After 24 and 48 hours of exposure, 10µL of MTT reagent (5mg/mL in 1x PBS PH 7.4) was added in each required well. The plate was wrapped with aluminum foil to avoid light exposure as MTT is light sensitive and incubated in a humidified incubator at 37^{o}C with 5% CO_2 for four hours. 100 µL of crystals solubilizing solution (isopropanol with 0.04 N HCl) was further added into each well and mixed properly by equal and repeated pipetting. Absorbance was taken at 570 nm as test wavelength and at 670 nm as reference wavelength using synergy HTX multi-mode reader. To remove non-specific absorbance values, absorbance at 670 nm was subtracted from the absorbance at 570 nm. Cells viability was determined using the following formula:

$$\% \text{ Cell Viability} = A \text{ (test)}/ A \text{ (control)} \times 100$$

Where A (test) means absorbance of treated wells and A (control) represents absorbance of untreated cells.

$$\% \text{ Cell Death} = 100 - \% \text{ Cell Viability}$$

DNA fragmentation assay

For the detection of DNA fragmentation, HCT-116 and THP-1 cells (1×10^6 cells/mL), were treated with different concentrations of plant extracts (25, 50, 75, 100 and 200µg/mL) for 24 and 48 h in 6 well plate. Afterwards, cells were centrifuged at 10,000 RPM for 10 minutes and washed with PBS.

DNA Isolation of HCT116 Cells

DNA isolation from HCT-116 cells was performed following a method employed by Yui *et al*. (2005) with few modifications. The cell pellet was suspended in 100µL of lysis buffer (10 mM Tris-HCl), 10 mM EDTA (pH 8.0), 0.5% Triton X-100) and incubated for 10 minutes at 4^{o}C. Following centrifugation at 13,000 RPM for 5 minutes, supernatant was transferred into another eppendorf, treated with 2uL of 1mg/mL RNase and incubated for 1 hour at 37^{o}C. Digestion of lysed cells was done by adding 2uL of proteinase K (20 mg/mL) and incubating for 30 minutes at 50^{o}C. Then centrifugation was done at 12000 RPM for 5 minutes and DNA containing supernatant was transferred to a clean sterilized eppendorf. The DNA diluted with 6X loading dye (1:1) was run at 80 V on 1.5% agarose gel having 0.5µg/mL EtBr using 1kb DNA marker. DNA bands were visualized under UV- trans illuminator.

DNA Isolation of THP-1 Cells

The protocol for DNA isolation from THP-1 cells was performed as done by Mirakabadi *et al*. (2012) with few modifications. The cells were suspended in 100ul of lysis buffer (10mM EDTA (pH 8.0), 50mM Tris-HCl, 0.5% SDS), treated with 2.5uL of proteinase K(20mg/mL) and incubated at 55^{o}C for 1 hour. This was followed by centrifugation at 13000 RPM, then transferring the supernatant to another clean eppendorf. After RNase treatment (200ug/mL), phenol (25): chloroform (24): Isoamyl alcohol (1) solution was used for the extraction of DNA in a volume equals to that of the supernatant for 1 min. Following centrifugation at 12000 rpm for 5 min, the extracted DNA was diluted with 6X loading dye (1:1) and run at 80 V on 1.5 % agarose gel having 0.5µg/mL EtBr using 1kb DNA marker. DNA bands were visualized under UV- transilluminator.

Table 1: Percent Inhibition (I %) of free radicals produced by DPPH by stem and leaf extracts of C. *diurnum* and *C. nocturnum*

Concentrations (ug/mL)	Percent Inhibition (I %)				
5000	CDS	CDL	CNS	CNL	A.A
	89.0±0.6	88.1±0.3	62.0±0.2	81.5±	81±0.1
1000	53±0.9	51±0.6	40±0.3	50±0.1	48.5±0.4
500	35±0.2	34±0.3	28±0.1	32±0.1	32±0.2
100	14±0.0	13.9±0.2	10±0.09	12.9±0.1	12±0.3
50	3±0.05	2±0.08	2.7±0.06	6.7±0.07	2±0.01

CDS: *C. diurnum* stem extracts CDL: *C. diurnum* leaf extracts CNS: *C. nocturnum* stem extracts CNL: *C. diurnum* leaf extracts A.A: Ascorbic acid (positive control)

Table 2: Percent % Cell death of HCT-116 and THP-1 cell lines after 24 and 48 hours exposure by *C. diurnum* and *C. nocturnum* extracts at five different concentrations

Plant extract	Conc.	% Cell death of HCT-116 after 24 h	% Cell death of HCT-116 after 48h	% Cell death of THP-1 after 24h	% Cell death of THP-1 after 48h
CDL	200	30.52	64.77	78.88	82.59
	100	19.26	32.39	39.11	56.33
	75	15.9	19.05	23.6	27.3
	50	3.6	5.72	5.62	16.74
	25	0.24	1.91	3.14	6
CDS	200	94.09	99.05	95.1	96.81
	100	93.16	94.29	88.2	91.266
	75	92.46	90.53	77.73	87.5
	50	68.68	78.02	76.9	81.64
	25	27.73	49.56	70.14	74.7
CNL	200	85.3	92.58	90.56	91.03
	100	67.89	90.48	81.12	80.01
	75	47.14	77.18	70.03	65.99
	50	35.69	54.29	41.26	52.4
	25	5.57	41.77	24.59	42.9
CNS	200	92	97.15	93.73	94.97
	100	90.14	92.28	87.3	89.05
	75	88.75	88.06	73	85.05
	50	65.56	76.06	68.7	75.06
	25	20.89	43.67	60.05	62.44

CDS: *C. diurnum* stem extracts CDL: *C. diurnum* leaf extracts CNS: *C. nocturnum* stem extracts CNL: *C. diurnum* leaf extracts

DPPH assay

The antioxidant activities of all extracts were performed using DPPH (2,2-diphenyl-1-picryl-hydrazyl-hydrate) free radical scavenging assay (Al-Reza, Rahman et al. 2009). For antioxidant activity, 100mL of 0.004% DPPH solution as well as 5000, 1000, 500, 100, 50ug/mL dilutions of CDS, CDL, CNL, CNS were prepared in methanol. Freshly prepared DPPH (2.9mL) was taken in clean and dried test tubes and 100uL of each dilution was added into it. After 1hr incubation at room temperature, absorbance was measured at 517 nm against blank (methanol). Ascorbic acid (with same dilutions in injection water) was used as standard. Percent inhibition of free radicals produced by DPPH was calculated as follows:

$$\text{Percent inhibition (I \%)} = \times 100$$

Where A (control) is the absorbance of DPPH without test compound and A (test) is the absorbance of DPPH with test compound.

STATISTICAL ANALYSIS

Absorbance values that were lower than the control wells indicated a reduction in the rate of cell proliferation. Conversely, a higher absorbance value indicated an increase in cell proliferation. Rarely, an increase in proliferation might be offset by cell death; evidence of cell death was inferred from morphological analysis. Results were expressed as mean ± SD of experiments done in triplicates by using software two way ANOVA version 6 to determine the significance of difference.

RESULTS

Antioxidant Activity

Antioxidant activity of stem and leaf extracts of *C. diurnum* (CDS and CDL) and *C. nocturnum* (CNS and CNL) was evaluated by DPPH free radical scavenging method while using ascorbic acid as a positive control. Percent Inhibition (I %) of free radicals produced by

DPPH by the plant extracts and ascorbic acid was determined (Table 1). Comparing the antioxidant activity of all test samples, stem extracts of *C. nocturnum* showed less antioxidant activity whereas leaf extracts of *C. nocturnum* showed antioxidant activity comparable to that of positive control, while stem and leaf extracts showed antioxidant potential even more than that of ascorbic acid (table 1, fig.1).

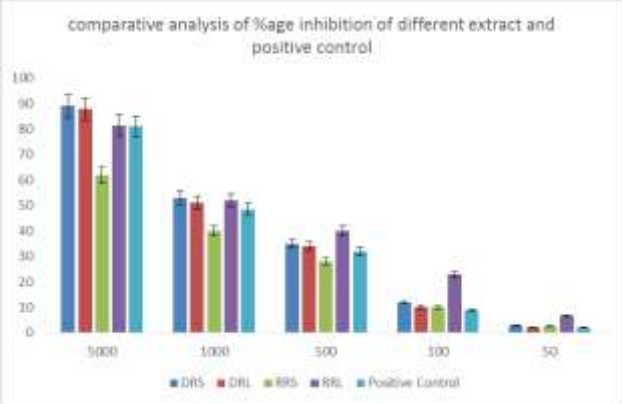

Fig. 1: Comparative percentage inhibition of free radicals by plant extracts and A.A as positive control. CDS: *C. diurnum* stem extracts, CDL: *C. diurnum* leaf extracts, CNS: *C. nocturnum* stem extracts CNL: *C. diurnum* leaf extracts and A.A: Ascorbic acid (positive control).

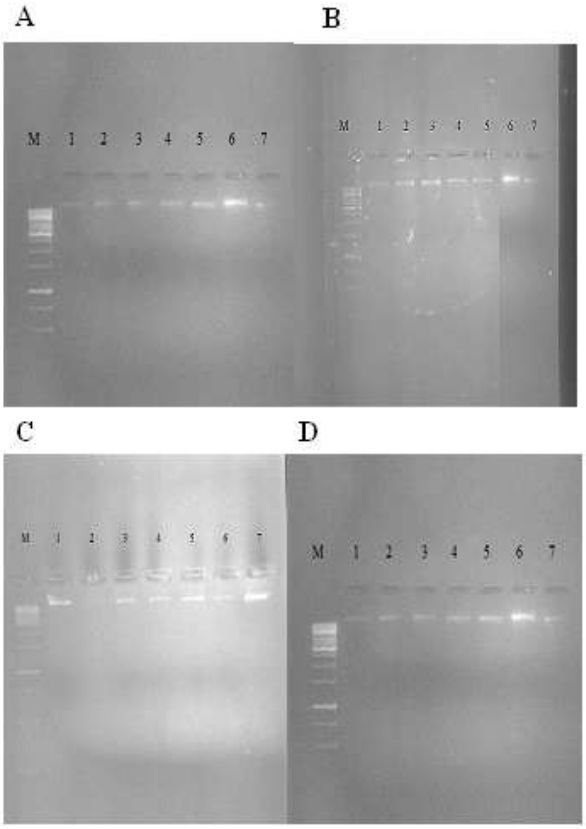

Fig. 2.1: Effect of different concentrations of extracts of CNL (A), CNS (B), CDL (C), CDS (D) on DNA of HCT-116 cells after 24 hours treatment along with positive and negative controls Lane M: 1Kb ladder, Lane 1-5: (200µg/ml, 100µg/ml, 75µg/ml, 50µg/ml, 25µg/ml respectively) Lane 6: Positive control, Lane 7: Negative control

Anticancer Activity

Percent viability was determined after exposing the HCT-116 and THP-1 cell lines to five different concentrations ((200µg/mL, 100µg/mL, 75µg/mL 50µg/mL, 25µg/mL) of leaf and stem extracts of *C. nocturnum* and *C. diurnum.* The results showed that all the extracts caused cell death in a dose dependence manner (Table 2). This means that cell death occurred in the order of 200µg/mL > 100µg/mL > 75µg/mL > 50µg/mL > 25µg/mL. After comparing percent cell viability, caused by different extracts it was also observed that all concentrations of extracts cause cell death in the following order:

CDS > CNS > CNL > CDL

Moreover, anticancer activity of the extracts was also increased with the increase of their duration of exposure to cells.

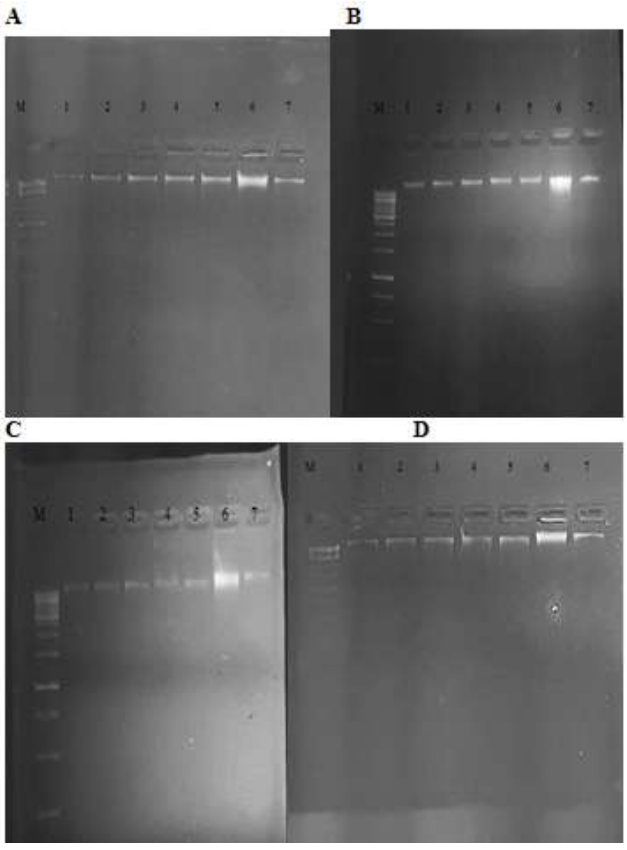

Fig. 2.2: Effect of different concentrations of extracts of CNL (A), CNS (B), CDL (C), and CDS (D) on DNA of HCT-116 cells after 48 hours treatment along with positive and negative controls Lane M: 1Kb ladder, Lane 1-5(200µg/ml, 100µg/ml, 75µg/ml, 50 µg/ml, 25 µg/ml respectively) Lane 6: Positive control, Lane 7: Negative control.

DNA fragmentation assay

In DNA fragmentation assay, two cell lines e.g., (HCT-116 and THP-1) were exposed with five different concentrations (200µg/mL, 100µg/mLl, 75µg/mL, 50µg/mL, 25µg/mL) of CDS, CDL, CNS and CNL for 24 and 48 hours by using 3 mM H_2O_2 as positive control and without treatment cells as negative control. The cells palletization DNA of the cells were isolated and visualized after agarose gel electrophoresis. The assay was performed to determine that either the plant extracts has damaged the DNA or not. And if yes then either it is due to apoptosis leading to 180bps DNA fragments or necrosis making a smear on gel.

It was observed that no degradation was there in DNA of HCT-116 given 24 hours of plant extracts exposure even not for the positive control as shown in figs. from (fig. 2.1). Similarly DNA of HCT-116 cells given exposure of plant extracts for 48 hours showed approximately no degradation except for the positive control which undergone some extent of degradation (fig. 2.2). But DNA of THP-1 cells when given 24 hours of extracts exposure showed very little or no degradation while THP-1 cells given 48 hours of plant extracts exposure undergone from mild to severe degradation (fig. 3.1, 3.2). Leaf and stem extracts of *C. nocturnum* (CNS and CNL) showed mild degradation as compare to the leaf and stem extracts of *C. diurnum* (CDS and CDL) which showed more degradation.

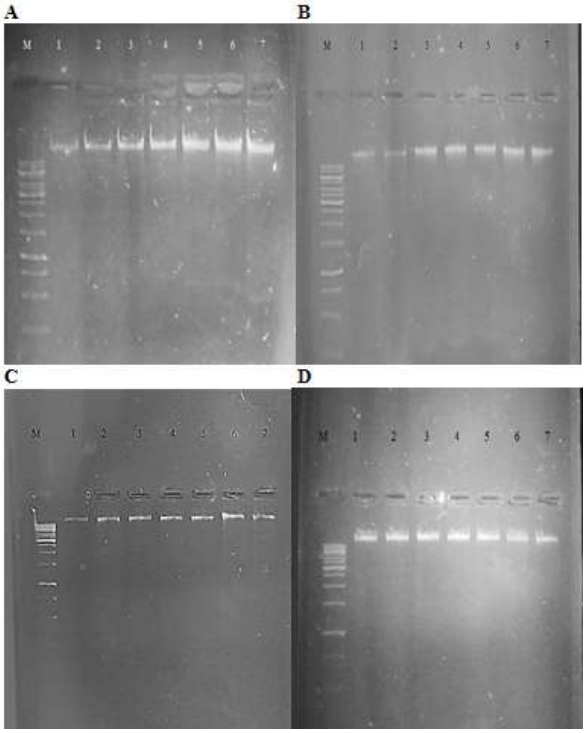

Fig. 3.1: Effect of different concentrations of extracts of CNL (A), CDL (B), CNS (C), CDS (D) on DNA of THP-1-1 cells after 24 hours treatment along with positive and negative controls Lane M: 1Kb ladder, Lane 1-5(200µg/ml, 100µg/ml, 75µg/ml, 50µg/ml, 25µg/ml respectively) Lane 6: Positive control, Lane 7: Negative control

DISCUSSION

Pharmacological significance of plants can be attributed to the therapeutic nature of their biologically active phytochemicals or secondary metabolites which include phenolic compounds, terpenoids, alkaloids, saponins and tannins (Joseph and Raj, 2010). Phenolic compounds including flavonoids and tannins are good antioxidants because of their free radicals scavenging ability. They perform the activity by quenching singlet oxygen, chelating metal ions, acting as reducing agents or donating electrons which may vary depending upon their structures (Gulcin, Bursal *et al*. 2010; Chang *et al*. 2002 and Zhang *et al*. 2013). These compounds are also responsible for the anticancer properties of plants (Prasad *et al.* 2013). The reason is that these antioxidants scavenge the radicals causing carcinogenic DNA mutations (Bellion *et al*. 2010). Alkaloids, flavonoids, tannins and terpenoids also exhibit antimicrobial properties. Saponins are associated with cholesterol lowering, immunity enhancing, tumor suppressing as well as anti-inflammatory activities (Waller and Yamasaki 2013) *C. nocturnum* and *C. diurnum* also possess such medicinally important metabolites making them suitable to be used in a variety of applications. Methanol extracts of these plants were screened for alkaloids, tannins, flavonoids and terpenoids by Prasad *et al*. (2013).

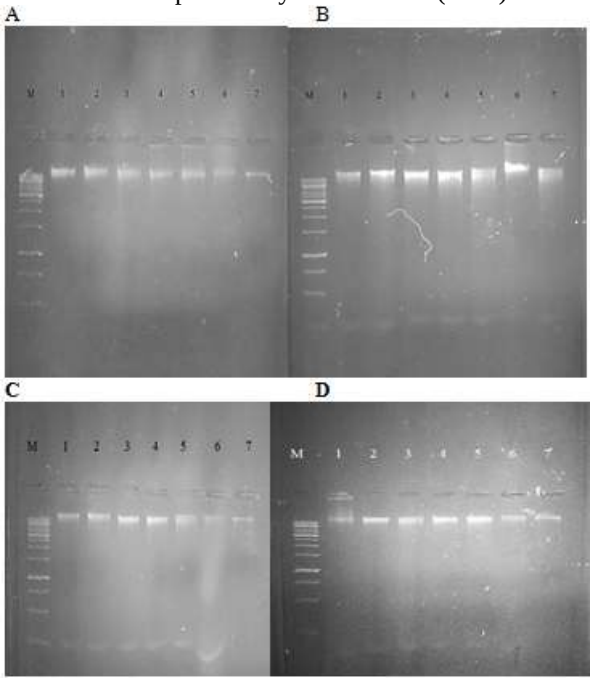

Fig. 3.2: Effect of different concentrations of extracts of CNL (A), CNS (B), CDS (C), CDL (D) on DNA of THP-1-1 cells after 48 hours treatment along with positive and negative controls Lane M: 1Kb ladder, Lane 1-

5(200µg/ml, 100µg/ml, 75µg/ml, 50µg/ml, 25µg/ml respectively) Lane 6: Positive control, Lane 7: Negative control.

In the present study, antioxidant and anticancer properties of the stem and leaf extracts of the plants were evaluated. Antioxidant activity was determined by a sensitive DPPH free radical scavenging method. Leaf and stem extracts of *C. diurnum* showed more antioxidant potential as compare to the extracts of *C. diurnum* and the results were according the previous study (Prasad *et al*. 2013). The overall order of the antioxidant potential of the extracts at 5000ug/mL was as follows: CDS (89%) > CDL (88%) > CNL (81.5%) > CNS (62%). Moreover, some of them showed the antioxidant potential greater than that of the positive control (ascorbic acid) with % inhibition of free radical was 81%. Exact mechanism is still not known but this may be due to the difference of metabolites in the extracts which have altered this activity. However, all the extracts showed the antioxidant potential in a dose dependent manner which was due to the increased content of the phytochemicals in higher dose of the extracts.

Anticancer activity of the different concentrations (200µg/mL, 100µg/mL, 75µg/mL, 50µg/mL, and 25µg/mL) of CNS, CNL, CDS and CDL extracts was also investigated. The experiment was performed on HCT-116 and THP-1 cell lines as they were easily available in the laboratory belong to the Risk group 1 in terms of bio-safety level as well as had never previously studied for these extracts. The anticancer activity was investigated as % cell death which was determined by MTT assay. The percentage cell death was determined after 24 and 48 hours of exposure by plant extracts. The results exhibited that the extracts showed anticancer activity in time and dose response manner since more cell death was observed as the concentration of the extracts and duration of extracts exposure was increased. The results were similar to the previous studies (Mimaki, *et al*. 2002; Zhao *et al*. 2008 and Luo *et al*. 2009) which reported the anticancer activity of different extracts of *C. nocturnum* against different cell lines. The order with which the extracts showed cell death was CDS > CNS > CNL > CDL. Further, CDS extracts were found to be more effective than other extracts which might be attributed to their phytochemical content while the extracts were active, thus showing anticancer potential against both types of cell lines somehow in the same way.

Cell death can be correlated with DNA damage as it can results into apoptosis or necrosis (Zhivotosky and Orrenius, 2011). Apoptosis is associated with the formation of cleaved DNA fragments of 180 bps forming DNA ladders on agarose gel while necrosis occur accidentally due to some stress conditions, energy depletion or may also arise in cancerous cells in response to some alkylating agents causing DNA damage (Gorman *et al*. 2010) forming smear on agarose gel (Zhivotosky and Orrenius 2001). Since cell death can be due to the DNA damage, so effect of the extracts on DNA damage was also investigated by isolating the DNA of all cells exposed to the different concentrations of all extracts (CDS, CDL, CNS, CNL) at two time points (24 h and 48 h). The isolated DNA samples were electrophoresed in agarose gel (1.5% with EtBr) at 80 volts and visualized under UV light. It was observed that no degradation was there in DNA of HCT-116 given 24 hours of plant extracts exposure even not for the positive control as shown in figs. from (figs. 2). Similarly DNA of HCT-116 cells given exposure of plant extracts for 48 hours showed approximately no degradation except for the positive control which undergone some extent of degradation (fig. 3). But DNA of THP-1 cells given 24 hours of extracts exposure showed very little or no degradation while THP-1 cells given 48 hours of plant extracts exposure undergone from mild to severe degradation (fig. 4-5). Leaf and stem extracts of *C. nocturnum* (CNL and CNS) showed mild degradation as compare to the leaf and stem extracts of *C. diurnum* (CDL and CDS) which showed more degradation. In the experiment positive control (3mM H_2O_2) was used based on its DNA fragmentation activity as studied by Sun *et al*. (2012) against H5V (murine heart micro vessel endothelial) cell line. But might be their amount against HCT-116 and THP-1 cell line is too low that could not cause DNA ladder formation even after 48 hours of exposure or had affected the DNA in some other manner that degraded the DNA in a way forming its smear on agarose gel. Moreover, no degradation was observed in both types of cells after 24 hours of extracts exposure. Actual cause is not known but we can hypothesize that this might be due to the reason that DNA was not affected until after 24 hours of exposure or due to some other unknown reason. DNA smear formation in case of THP-1 cell lines after 48 hours of extracts exposure showed that the cell death was might be due to necrosis.

CONCLUSION

In conclusion, the results presented in this paper demonstrate that *C. noctornum* and *C. diurnum* methanolic leaves and stems extracts have remarkable antioxidant and anticancer activities which not only verify their medicinal value but also encourage their use as potent antioxidant and anticancer agent.

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