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Antioxidant and cytotoxic effects of hexane extract of *Morinda pubescens* leaves in human liver cancer cell line

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ABSTRACT

Objective: To evaluate the antioxidant and cytotoxic effects of hexane extract of *Morinda pubescens* leaves and to find the primary bioactive compound responsible for antioxidant and cytotoxic activities. **Methods:** The individual compounds were isolated using column chromatography and were characterized by spectroscopic techniques. The antioxidant activity was evaluated for all individual isolated compounds by DPPH method using L-Ascorbic acid as standard and cytotoxicity was assessed for the extract and the hyoscyamine by MTT assay, caspase test and RT-PCR study. **Results:** The antioxidant activity of the isolated compounds and the extract increased as the concentration increased. One of the isolated compound hyoscyamine showed the high antioxidant activity. The extract and the hyoscyamine dose-dependently decreased the cell viability in HepG2 cells. Hyoscyamine induced caspase-3 mediated apoptosis. Up regulation of p53 gene expression provides cue for apoptotic activity of hyoscyamine. **Conclusions:** The results indicate that hexane extract possessed potent antioxidant and cytotoxic activity and hyoscyamine is the principal bioactive compound in hexane extract.

1. Introduction

The imbalance between the generation and the neutralization of reactive oxygen species by antioxidant mechanisms within an organism is called oxidative stress[1]. Oxidative stress is now recognized to be associated with more than 100 diseases, as well as with the normal aging diseases like stroke, diabetes, cancer, cardiovascular diseases, AIDS and neuro generative diseases such as Alzheimer's and Parkinsonism *etc*[2]. Oxidative stress has also been recognized to be involved in the etiology of liver diseases[3]. Hepatocellular carcinoma is the fifth most common cancer and the third most common cause of cancer-related death[4,5].

Plants have a long history in the treatment of different cancer cells[6]. Natural antioxidants have been proposed and utilized as therapeutic agents to counteract liver damage[7]. Many species of *Morinda* genus have been reported for various health disorders and anticancer activity by Indian pharmacopoeia. For instance, *Morinda critifolia* which is also called Noni or Yor contain several medicinally active components that exhibited various therapeutic effects. These include anti-bacterial, anti-viral, and anti-cancer activities as well as analgesic effects[8, 9]. Antitumour potential from the fruit of *Morinda citrifolia* on sarcoma 180 ascites tumour were reported[10,11]. Cytotoxic anthra quinones were isolated from *Morinda parvifolia*[12]. *Morinda tinctoria* was reported for its anti ulcer activities[13]. The antibiotic and anti-inflammatory activities of leaves of *Morinda pubescens (M. pubescens)* were studied[14]. However, scientific literature data supporting the folkloric use of *M. pubescens* in cancer studies are not available and its tentative mechanism(s) are still unknown. Hence the goal of present study was to screen the hexane extract of *M. pubescens* leaves for antioxidant potential, cytotoxic effect and caspase-3 and caspase-9 mediated apoptosis. Also to find the bioactive compounds that predominantly involved in anticancer activities.

2. Material and methods

2.1. Chemicals used

2, 2-diphenyl-1-picrylhydrazyl (DPPH), L-ascorbic acid was purchased from Sigma Chemical Co. U.S.A. All other

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reagents were of analytical grade and were used as received.

2.2. Instruments used

Absorption UV spectra were recorded with an systronics 2201 double beam spectrophotometer. FTIR spectra were conducted on a Perkin-Elmer spectrophotometer. The NMR spectra were recorded using an Advance 400 Bruker spectrometer (400.13 MHz for 1H, 100.61 MHz for 13C spectra). All spectra were acquired in $CDCl_3$ and chemical shifts are reported in ppm (d) relative to residual solvent peaks (dH 7 and dC 77.6) with TMS as an internal reference. The EI-MS mass spectrum was recorded on a JEOL GC-mate mass spectrophotometer.

2.3. Preparation of plant extract

M. pubescens Smith belonging to the family of Rubiaceae was collected in Auxilium College, Vellore, Tamil Nadu, India. The species was identified and authenticated by Ms. Isabella Roseline, Head, Department of Botany, Auxilium College and the Vouchers of the plant specimen were deposited in the Department of Botany, Auxilium College with the code DRC_mp1.

2.4. Extraction and fractionation procedure

Leaves of the plant (3.5 kg) were shade dried, pulverized and percolated in *n*-hexane thrice. The filtrate was concentrated at 40 ℃ under reduced pressure by a rotary vacuum evaporator (Super fit, Chennai, India) to give a semisolid residue of approximately 63 g.

2.5. Isolation of individual pure compounds

The concentrated extract was column chromatographed over silica gel (60-120 mesh). It was eluted with different eluent mixtures like 100% hexane, hexane/diethyl ether, hexane/chloroform and hexane/ethyl acetate to give subfractions. The fractions were tested for its purity using TLC and the structures were characterized by UV, IR, EI-MS, ^{1}HNMR and ^{13}CNMR. The structures obtained were confirmed by comparing with the literature data[15] and the individual compounds are stigmasteroid, ergosteroid, E-phytol, campesta-5-22-trien-3-ol, stigmasta-4-en-3-one, stigmasta-4-22-dien-3-one, β-sistosterol and an alkaloid hyoscyamine.

2.6. DPPH-Antioxidant assay

Antioxidant activities of the leaves of *M. pubescens* in n-hexane extract and its isolated individual compounds were studied by DPPH method using a standard procedure[16] at 517 nm. The percentage of inhibition was calculated and was compared with standard L-ascorbic acid.

2.7. Cell culture

Suspension target cell line, hepatocellular carcinoma (HepG2) was obtained from National Centre for Cell Science, Pune, India, and was maintained in Duelbacco Minimum Eagle's Medium with 10% Fetal Bovine Serum at 37 β, 5% CO_2 and 90% humidity throughout the study. The cell viability was assessed by the 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphynyltetrazolium bromide (MTT) assay.

2.8. MTT Assay

To determine cell viability, cell number was quantified using the standard Colorimetric MTT assay[17]. The colorimetric assay is based on the conversion of the yellow tetrazolium bromide to the purple formazan derivatives by mitochondrial succinate dehydrogenase in viable cells. MTT was dissolved in 0.01 M phosphate buffered saline at 5 mg/mL and stored at 4 ℃. Cells were seeded overnight in 96-well culture plates at a density of 5×10^3 cells/well with 100 μL culture media. Cells were treated with n-Hexane extract and the isolated compound hyoscyamine at different concentrations like 25, 50, 100 and 250 μg/mL. The solvent DMSO was dissolved in culture media. After 24 hrs of incubation, 20 μL of 5 mg/mL MTT was added to each well and incubated for an additional 4h at 37 ℃. Then 80 μL of SDS/HCl solution was added to the wells to solubilize the MTT crystals. The plates were incubated overnight at 37 ℃. The potency of cell growth inhibition for each extract was expressed as IC_{50} value. The plate was read for optical density at 570 nm, with reference wavelength of 620 nm using a plate reader. The dry crude extracts were dissolved in DMSO, and then diluted in 5:100 in cell culture medium before preparing the indicated concentrations. Viability was defined as the ratio (expressed as percentage) of absorbance of treated cells to untreated cells[18]. Percent cytotoxicity was calculated after comparing with the untreated control. The cyclophosphamide was used as a standard drug.

2.9. Determination of caspase activity

Caspase-3-activity was monitored by the cleavage of Ac-Asp-Glu-Val-Asp-p-nitoranilide (DEVD-pNA) according to the protocol outlined by the manufacturer in a caspase-3 and Colorimetric Protease Assay Kit (Promochem, USA) and Ac-LEHD.pNA for caspase-9. After treatment with designated concentrations of extract at concentrations 25-100 μg/mL, cell lysates were prepared by incubating 2×10^6 cells/mL in cell lysis buffer for 10 min in ice. Lysates were centrifuged at 10 000 rpm for 1 min. The supernatants were collected and protein concentration was determined by the Bradford's method using BSA as a standard[19]. About 100-200 μg protein was diluted in 50 μL cell lysis buffer for each assay. Cellular extracts were then incubated in 96-well microtiter plates with 5 μL of the 4 mM p-nitroanilide (pNA) substrates, DEVD-ala-pNA for caspase-3 activity and LEHD.pNA for caspase-9 for 2 h at 37 ℃. The relative caspase-3 and 9 activities were calculated as a ratio of absorbance of treated cells to untreated cells.

2.10. Reverse transcription-polymerase chain reaction analysis

Total RNA was isolated with One-step RNA Reagent purchased from Bio Basic Inc, Canada and spectrophotometrically quantified. The RT reaction was performed with 5 μg of total RNA and an oligo primer using the First-Strand cDNA synthesis kit purchased from Applied Biological Material Inc, Canada according to the manufacturer's instruction and the experiment was carried out by the standard procedure[20]. The primers used are: (F) 5'GAAGACCCAGGTCCAGATGA 3' (R) 5'CTCCGTCATGTGCTGTGACT 3' and GAPDH.

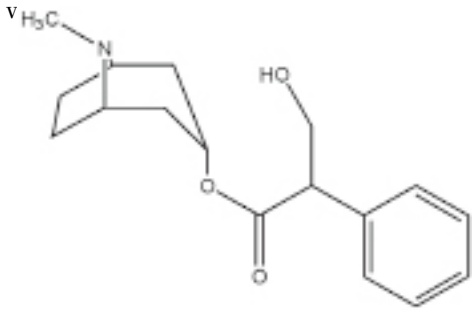
2.11. Statistical analysis

The results were expressed as mean±SD of three independent experiments. The statistical analysis involving two groups was performed by means of Student's *t*-test, whereas analysis of variance (ANOVA) followed by Dunnett's multiple comparison test were used in order to compare more than two groups. The difference at $P<0.05$ was considered significant and $P<0.001$ was considered more significant. All data were processed using SPSS -12.

3. Results

3.1. Characterization of hyoscyamine

UV λ_{max} 212. IR (KBR) ν_{max}/cm^{-1} 3433br (OH), 2936(CH), 1725(C=O), 1640(C=C aromatic), 1240 (C-O ether linkage), 1023 (aliphatic amine CN), 970 (Vinyl oops). MS m/z (rel.int): 289.0094[M^+], calculated for $C_{17}H_{23}NO_3$[21]. 1H ($CDCl_3$, 500MHz), δ 7.1307 (2H, t), δ 7.5296 (2H, t), δ 7.2545 (1H, d), indicate the presence of phenyl ring, δ 3.57 (1H, q, J=5.3Hz) presence of OH group, δ 1.02-1.303(6H, broad) presence of pyrrolidine group, δ 1.968-2.090 (7H, multiplet) presence of piperidine group. In ^{13}C NMR, the quaternary carbon signal at 179.86 is characteristic of Carbonyl group. In addition, the signals at 123,130.1125 and 121.71 were assigned as substituted aromatic ring. There are chemical shifts of two methylene groups at 29.6930 (C7 and C8) and two methane groups at 77.3656. From the above spectral studies it was confirmed that the isolated compound is hyoscyamine belonging to an alkaloid group and its structure is given as:



3.2. Antioxidant activity

The antioxidant activity of the extract and its individual isolated compounds at different concentrations like 100, 200, 300, 400, 500, 600, 700 and 800 μg/mL are studied by DPPH method. The results are given in Table 1. The antioxidant activity increased as the concentration increased. Among the isolated compounds hyoscyamine showed the high antioxidant activity than the other individual compounds.

3.3. Cytotoxic activity

MTT assay was used as an indirect measure to determine the viability of HepG2 cells exposed to the hexane extract and hyoscyamine and the results are given in Figure 1. Both extract and hyoscyamine caused cell death in a concentration dependant manner.

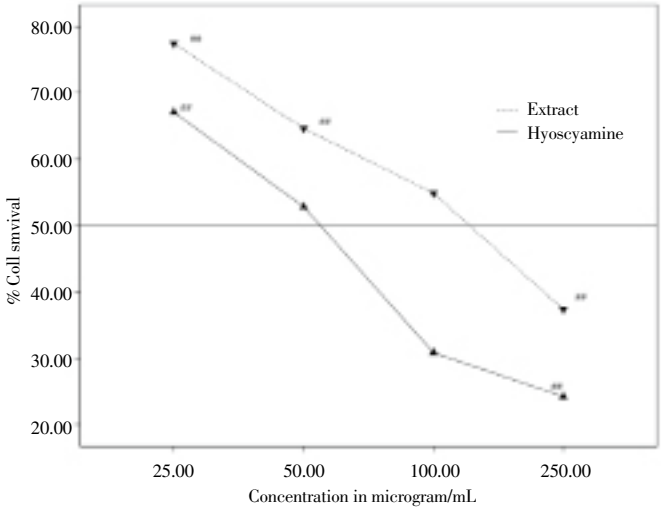


Figure 1. Cytotoxic effects of hexane extract and hyoscyamine on HepG2 cells.
% Cell survival is plotted against the concentration of extract.
Results are average of three replications. ## denotes that the values are significantly different ($P<0.001$) compared with control.

3.4. Induction of apoptosis by activating caspase-3 and caspase-9

Caspases are believed to play a central role in mediating apoptotic responses. To monitor the enzymatic activity of caspases during hyoscyamine-induced apoptosis, we used peptide substrate: DEVD-pNA and Ac-LEHD.pNA as substrate. Caspase activities were measured following treatment of HepG2 cells at different concentrations of

Table 1
Antioxidant activities of *n*-hexane extract and its individual isolated compounds in terms of IC_{50}.

S. No	Name of the compound	IC_{50}(μg/mL)	Antioxidant activity (%)
1	*n*-hexane extract	150.00±2.00	92.90
2	β-sistosterol	783.33±20.82	52.54
3	Campesta-5-22-trien-3-ol	731.33±29.00	55.56
4	Hyoscyamine	289.33±62.14	58.40
5	Stigmasteroid	>800	44.51
6	Ergosteroid	>800	44.10
7	E-phytol	>800	37.78
8	Stigmasta-4-en-3-one	>800	41.27
9	Stigmasta-4-22-dien-3-one	>800	40.89
10	L-ascorbic acid (Standard)	28.40	96.00

Each value represents the mean ± SD of three independent measurements.

hyoscyamine. From the Figure 2 it was evident that the activities of caspase-3 and caspase-9 increased as the concentration of hyoscyamine increased.

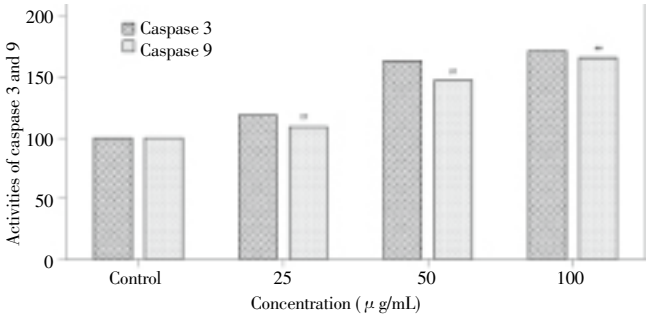


Figure 2. Effects of hyoscyamine on the caspase-3 and caspase-9 activity at different concentrations.
Cells were treated with hyoscyamine at 0, 25, 50 and 100 μg/mL for 24 h. After treatment, the cells were lysed; caspase-3 and caspase-9 activity of supernatant was measured. n=3, ##P<0.001, compared with the control.

3.5. Reverse transcriptase polymerase chain reaction (RT-PCR)

In this study we demonstrate for the first time that hyoscyamine which is an alkaloid modulated caspase-3 to trigger apoptosis signaling that mediates the growth inhibition of HepG2. As seen in Figure 3, Full length cDNA was subjected to amplification using p53 primers and compared with the treated cell line (L4) with untreated cells line (L3). The p53 gene was up expressed at high levels than untreated cells. Hyoscyamine enhanced p53 gene expression in HepG2 cells fourfold induction when compared to untreated cultures in HepG2.

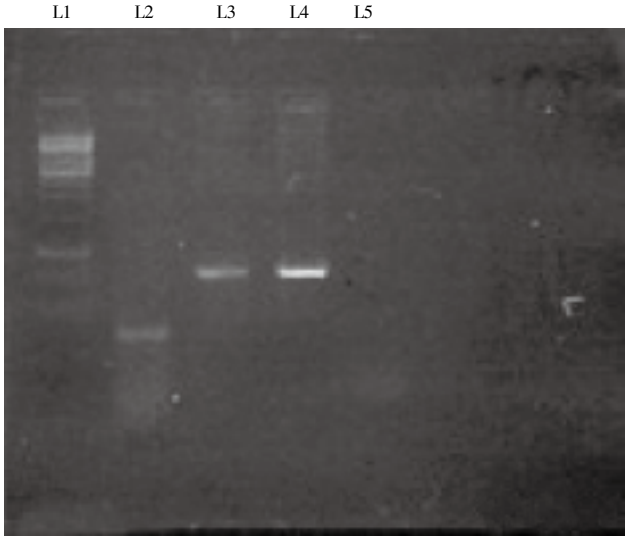


Figure 3. RT-PCR photograph of hyoscyamine.
L 1:1 kb ladder, L 2: Positive control for Rt, L 3: Untreated cell line, L 4: Treated cell line, L 5: Negative control for Rt.

4. Discussion

Human cells are constantly exposed to reactive oxygen radicals generated by a number of biotic and abiotic factors such as irradiation, environmental factors, pollutants, stress or by products of metabolic processes. When the exposure overwhelms endogenous preventive systems, cells are exposed to potentially harmful load of oxidants, leading to various free radicals induced noxious effects. Free radical attacks biological molecules such as lipids, proteins, enzymes, DNA and RNA leading to cell or tissue injury associated with many diseases including ageing, atherosclerosis, heart diseases and carcinogenesis *etc*[22]. Antioxidants are compounds which act as radical scavengers when added to the food products and prevent the radical chain reaction of oxidation, delay or inhibit the oxidation process and increase shelf life by retarding the processes of lipid peroxidation[23]. Thus the antioxidant activities of the combined extract and the eight individual isolated compounds were studied. The hexane extract showed the highest antioxidant activity of 93% at 800 μg/mL. The high antioxidant potential for the extract may be due to the combined effect of the individual phytochemicals present in the extract.

The antioxidant activities of the individual compounds decreased in the order: Hyoscyamine > campesta-5-22-trien-3-ol > β-sistosterol > stigmasteroid > ergosteroid > stigmasta-4-en-3-one > stigmasta-4-22-dien-3-one > E-phytol. Among the isolated compound hyoscyamine showed the high antioxidant activity when compared to other compounds and for this reason, hyoscyamine was considered for further studies.

During the past decades, the killing of tumors through the induction of apoptosis has been recognized as a novel strategy for the identification of anticancer drugs[24-27]. Apoptosis (Programmed cell death) originally referred to an active form of cell death with stereotypic morphological characteristics occurring during the development. A broad range of pathological conditions can induce apoptosis. Unbalanced cell proliferation and apoptosis may play a role in pathogenis of certain types of tumors and neurodegenerative diseases[28-32]. Our study showed that the Hyoscyamine showed the strongest cytotoxic (Induction of apoptosis) effect on HepG2 (78%) at 24 h when compared to extract whose IC_{50} value is 132 μg/mL. Hyoscyamine had a much smaller IC_{50} value (54 μg/mL) as compared to that of hexane extract, suggesting the former is more effective against HepG2 cell proliferation than the latter and they were compared with standard cyclophosmamide (95.3%).

Caspases present in mitochondria are the crucial mediators of apoptosis. Of the 14 caspases identified in mammals, caspase-3, previously called CPP32, Yama, apopain is the major downstream protease in all apoptotic pathways[33, 34]. The most notorious apoptogenic factor released from permeabilized mitochondria is the respiratory component cytochrome c, which recruits apoptosis protease activating factor called Apaf-1 and procaspase-9 to form apoptosome, caspase-9 is thus activated, and orchestrates caspase-3 and other effector molecules for the cell death[35]. From our study it was evident that, when hyoscyamine was added to the culture medium, a significant increase in the caspases -3 and caspases-9 protein levels were observed. Moreover, the dose-dependent up-regulation of caspases-3 and caspases-9 activation by hyoscyamine was confirmed.

The p53 pathway is preferentially used control the apoptosis machinery. Roy *et al* reported that epigallocatechin-3-gallate inhibited HepG2 cell proliferation and induced apoptosis via p53-dependent and fas-mediated pathways[36]. Alkaloids are main bioactive chemicals in nux vomica[37] and they are effective against different types of cancer. The present study clearly indicates that hyoscyamine one of the isolated compound from the hexane extract up regulates caspase-3 expression, which leads to an enhancement in apoptosis susceptibility. We also demonstrated here for the first time that the potentiation of caspase-3 expression by

hyoscyamine is mediated via p53-dependent pathway. The results indicate that hyoscyamine is the primary bioactive compound from the hexane extract and supports the further research and development of the bioactive ingredients from *M. pubescens* leaves as anticancer agents, especially against liver cancer.

Conflict of interest statement

We declare that we have no conflict of interest.

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