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# Antioxidant and anticancer studies of chloroform extract of *Morinda pubescens* leaf

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## Abstract

*Morinda pubescens* is used for the treatment of diabetes, liver diseases, wound healing, cancer etc and this study was aimed to screen the chloroform extract of *Morinda pubescens* leaves for biologically active compounds and to evaluate the antioxidant and anticancer potential against HepG2 cell lines. The antioxidant activity of extract and its isolated compounds were carried out using 2, 2 diphenyl-1-picrylhydrazyl assay using L- ascorbic acid as a standard. The tetrazolium salt 3-[4, 5 dimethylthiazol-2yl]-2,5diphenyl trazolium bromide was used to determine the cell viability against HepG2 cells and Caspase-3 and -9 carried out to find the mechanism of the cytotoxicity. Octonyl glucopyranose, the isolated bioactive compound was subjected to RT-PCR study to confirm the apoptotic activity. The results revealed that the chloroform extract and three isolated compounds namely phytol, Octonyl glucopyranose and hexonyl glucopyranose exhibited significant antioxidant activities with $IC_{50}$ values of 550, 758, 482 and 645µg/mL respectively. The Octonyl glucopyranose one of the isolated bioactive compounds decreased the cell viability in HepG2 cells dose-dependently and increased the caspase-3 and caspase-9 protein levels to 1.3 folds than the normal cells. Augmentation of p53 expression was observed in the Octonyl glucopyranose treated cells than the untreated cells. These findings might offer valuable insights into the mechanism of anti-cancer activity of Octonyl glucopyranose HepG2 cells.

**Keywords**: DPPH, MTT, Octonyl glucopyranose, antioxidant, caspases, Morinda pubescens

## Introduction

The liver, as key organ of metabolism and excretion, is constantly endowed with the task of detoxification. Hepatotoxicants, including viruses, fungal products, bacterial metabolites, minerals, environmental pollutants and chemotherapeutic agents can induce various disorders in the human organs [1]. Most patients diagnosed with Hepatocellular carcinoma have low recovery rates, and conventional and modified therapies currently available are rarely beneficial [2]. In view of severe undesirable side effects of synthetic drugs, there is growing focus to follow systematic research methodology and to evaluate scientific basis for the traditional herbal medicine which are claimed to possess Hepatoprotective activity.

Medicinal plants are important sources of new chemical substance that potentially have strong therapeutic effects. Most people living in developing countries are dependent on traditional medical practices for their primary health care and the higher plants are known to be the main source for drug therapy in traditional medicine [3]. *Morinda pubescens* of *Rubiaceae* family commonly known as "Nuna" is widely distributed throughout India and its leaves are used in the treatment of liver diseases, ulcer and to heal the wounds [4, 5]. Several species of *Morinda* genus are scientifically reported for anticancer activities [6-12]. The thorough literature survey shows that there is no scientific report on liver cancer using *Morinda pubescens.* Hence, the present study was aimed to evaluate the antioxidant potential of the eight isolated compounds from the chloroform extract of *Morinda pubescens* leaves and to study the cytotoxic effect of selected isolated compounds like phytol, Octonyl glucopyranose and hexonylglucopyranose based on their antioxidant activities and to choose an bio active compound Octonyl glucopyranose to further investigate anticancer activity of against HepG2 cells lines.

## Material and methods

### Chemicals

All chemicals were of analytical grade. Silica Gel 60, Silica gel 60 $F_{254}$ coated plates, solvents for the extractions and analytical procedures, chloroform, ethyl acetate, methanol and ascorbic acid were purchased from Merck (Darmstadt, Germany). DPPH radical (1,1-diphenyl-2-picrylhydrazyl) was purchased from Sigma Chemical Co.(St. Louis, Mo, USA). Mass spectra were carried out using JEOL-GC mate HREIMS instrument and the UV spectra were recorded using SYSTRONICS 2201 instrument. NMR spectra were studied using a Bruker FTNMR 500 spectrometer. Chemical shifts were expressed as ppm relative to the TMS. Deuterated chloroform was used as solvent for all the samples.

### Plant Extract

Fresh leaves of *Morinda pubescens* was collected in October 2009 in Auxilium college campus, Vellore, Tamil Nadu, India and was authenticated by Ms. Isabella Roseline, Head, Department of Botany, Auxilium College. Two sets of herbarium voucher specimens were mounted and one set was placed in the Department of Botany, Auxilium College with the code no DRC_mp1.and the other set has been preserved in our laboratory for future reference.

Extraction and isolation of Individual constituents of chloroform extract

Leaves of the *Morinda pubescens* (3.5kg) were shade dried, pulverized and percolated thrice in solvents like Hexane, Chloroform, Ethylacetate and aqueous Methanol. The chloroform fraction was suspended in distilled water and partitioned with hexane, ethylacetate and resulting chloroform portion was concentrated at 40 C under reduced pressure by a rotary vacuum evaporator (Super fit, Chennai, India) and got a semisolid residue of approximately 28.0g. The chloroform fraction was subjected to silicagel column (60-120 mesh) using chloroform-ethylacetate-methanol-water gradient system as an eluent and obtained approximately ten fractions. The fractions one through six upon purification yielded known plant fatty acids like, linoleic acid, caprylic acid, caproic acid, phytol, phytolacetate and artenol and the Fraction 7 and 8 were pooled again and purified by column chromatography using 230-400 mesh and eluted with a stepwise gradient mixture of chloroform-ethylacetate with the ratio of 8:2, 7:3 and 5:5 which yielded two subfractions of A and B. The subfractions 'B' which was approximately 233mg was further purified using preparative TLC plates (20x20cm) with chloroform-ethylacetate as developing solvents (1.8:1.2) to isolate Octonyl glucopyranose (82mg). Upon purification of subfraction 'A', hexonylglucopyranose were obtained. The isolated compounds were confirmed by characterizing using different spectroscopic techniques like UV, IR, GC-MS, 1DNMR and 2DNMR and the detailed spectra of potent bioactive compounds like phytol, Octonyl glucopyranose and hexonylglucopyranose are explained in detail.

## DPPH-Antioxidant assay

Different concentrations varying from 100 to 800µg/mL of chloroform extract and the isolated compounds were taken in different cuvettes. About 2mL of $6X10^{-5}$ of methanolic solution of 2, 2-diphenyl-1-picryl hydrazyl radical (DPPH) was added to these cuvettes and the absorbance were measured. The absorbance of the samples was measured at 517nm to calculate antioxidant activities of the extract and the isolated compounds using a standard equation [13].

3-(4, 5-Dimethylthiazol – 2-yl)-2, 5-diphynyltetrazolium bromide (MTT) assay

To determine cell viability, cell number was quantified using the standard Colorimetric MTT assay. Viability was defined as the ratio of the absorbance of treated cells to untreated cells[14-16] and Cyclophosphamide was used as positive control.

## Determination of Caspase activity

The Caspase - 3 activities was monitored by the cleavage of Ac-Asp-Glu-Val-Asp-p-nitoranilide (DEVD-pNA) according to the protocol outlined by the manufacturers and the Colorimetric Protease Assay Kit (Promochem, USA) was used for the study. The cleavage of Ac-LEHD.pNA was used for caspase-9 using the above said protocol. Caspase activity was measured by cleavage of the above substrates to free pNA. The cleaved substrates, free pNA were measured by noting the absorbance at 405nm in a microtiter plate reader [17, 18]. Relative caspase-3 and 9 activities were calculated as a ratio of absorbance of treated cells to untreated cells.

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 (dT) primer using the First-Strand cDNA synthesis kit purchased from Applied Biological Material Inc, Canada according to the manufacturer's instructions. The experiment was carried out by the standard procedure [19] and the primers used are: (F) 5 `GAAGACCCAGGTCCAGATGA 3` (R) 5`CTCCGTCATGTGCTGTGACT 3` and GAPDH.

# Results and Discussion

## Characterization of isolated compounds

## Characterization of phytol

UV $\lambda_{max}$ 220. IR (KBR) $\nu_{max}/cm^{-1}$ 3433*br* (-OH), 2936(CH), 970(Vinyl oops). MS m/z (rel.int): 298.55[$M^+$], calculated for $C_{20}H_{42}O$. $^1H$ ($CDCl_3$, 500MHz), 4.08(1H, s) indicate the presence of OH group, 3.57 (2H, t) presence of methylene group adjacent to OH, -1.02-1.303(6H, broad) is due to long aliphatic chain, -1.4 (15H, dd) was assigned to terminal methyl groups. In $^{13}C$ NMR, Peak at -62.4 indicates the presence of oxygen linkage. All these spectral data confirm phytol and its IUPAC name is 3,7,11,15- tetra methyl hexa decan-1-ol. The proposed structure is given in figure 1a.

## Characterization of Octonyl glucopyranose

In the $^1H$ and $^{13}C$ NMR signal indicated the octanoyl partial structure. In the $^1H$NMR spectrum of Octonyl glucopyranose, two anomeric proton signals at -5.45 ((1H, d,*)* J -7.8 Hz) and 4.31 ((1H, d ) J-7.8 Hz) were observed. The $^{13}C$NMR also displayed signals at -104.5 (d), 77.9 (d), 77.9 (d), 75.0 (d), 71.4 (d), and 62.6 (t), attributable to terminal *β*-D-glucose and signals at -95.5 (d), 77.7 (d), 77.7 (d), 73.8 (d), 70.8 (d), and 69.4 (t) for the inner glucose unit. The 1-6 linkage of two glucose units present in *Octonyl glucopyranose* and the octanoyl moiety was present on the anomeric carbon of the central glucose as reported [20]. From the above evidence it was identified as 6-*O*-(β-D-glucopyranosyl)-1-*O*-octanoyl-β-D-glucopyranose. The structure was confirmed by HMBC spectra and the HMBC experiment showed the correlation contours between H-1 of the central glucose ( - 5.45) and the carbonyl carbon of the octanoyl moiety ( - 174.1), and between H-

1 of the terminal glucose ( - 4.31) and C-6 of the central glucose ( -69.4). The proposed structure of the compound is given as 1b

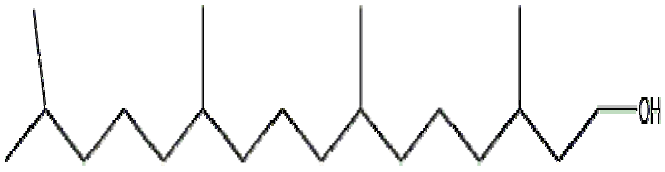
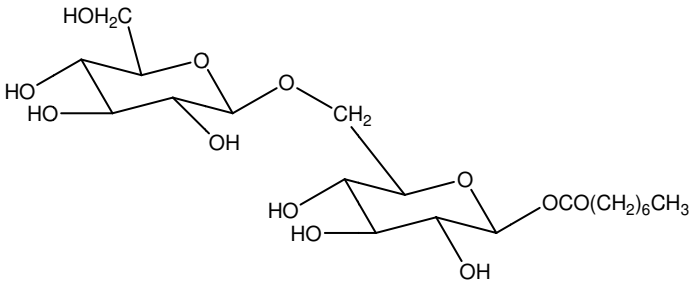


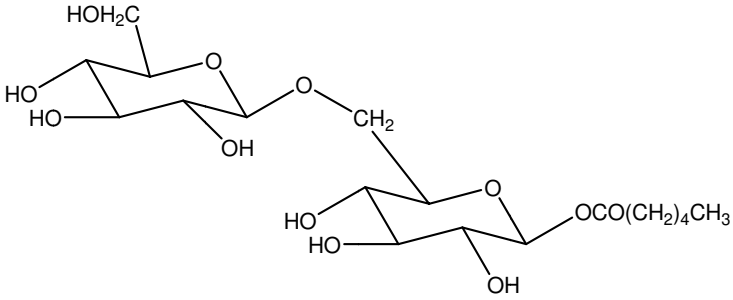

**Figure.1a Structure of phytol**

IUPAC name: 3,7,11,15- tetra methyl hexa decan-1-ol




IUPAC name: 6-*O*-(β-D-glucopyranosyl)-1-*O*-octanoyl-β-D-glucopyranose

**Figure.1b Structure of Octonylglucopyranose**




IUPAC name: 6-*O*-(*β*-D-glucopyranosyl)-1-*O*-hexanoyl-*β* –D glucopyranose

**Figure.1c Structure of hexonyl glucopyranose**

## Characterization of Hexonyl glucopyranose

*Hexonyl glucopyranose* was also obtained as a white powder. The HREIMS exhibited a significant molecular ion peak at *m/z* 440. These MS data together with the $^{1}H$ and $^{13}C$ NMR data, suggested the molecular formula as $C_{18}H_{32}O_{12}$. The IR spectrum showed hydroxyl and carbonyl absorptions at 3400cm$^{-1}$ and 1732cm$^{-1}$. In the $^{1}H$ NMR spectrum, signals at - 3.2 to 5.4 and in $^{13}C$NMR signals at – 75 and 77, showed similar to those of *Octonyl glucopyranose*. Only slight differences were observed in the high field where, instead of the signals for an octanoyl moiety, a signal for a hexanoyl moiety was observed. This observation was further supported by the $^{13}C$ NMR spectrum, which showed signals at - 14.3 (q), 23.4 (t), 25.3 (t), 32.3 (t), 34.8 (t), and 174.1 (s), assigned to a hexanoyl moiety [20]. The $^{1}H$NMR signals for the two anomeric protons were observed at - 5.45 and 4.31. Analysis of the HMBC spectra led to assignment of all $^{1}H$ and $^{13}C$ NMR signals for *Hexonyl glucopyranose*. Thus the compound was identified as 6-*O*-(*β*-D-glucopyranosyl)-1-*O*-hexanoyl-*β* –D glucopyranose and the structure is given as 1c

## Antioxidant activity by DPPH assay

The antioxidant activities of the extract and the individual compounds are given in table1.

**Table.1. Antioxidant potential of Extract and its isolated compounds of *Morinda pubescens***

| Compound | Antioxidant Potential (%) | IC50(µg/mL) |
|---|---|---|
| Chloroform Extract | 64.4±0.50 | 550 |
| Phytolacetate | 48.6±0.91 | >800 |
| Phytol | 51.3±1.01 | 758.33 |
| Octonyl glucopyranose | 64.6±0.64 | 482 |
| Hexonyl glucopyranose | 62.2±1.65 | 645 |
| Artenol | 58.8±0.23 | 644 |
| Linoleic acid | 56.2±0.29 | 655 |
| Caproic acid | 60.2±1.08 | 658 |
| Caprylic acid | 53.6±0.54 | 700 |
| L-Ascorbic acid | 92.0±1.41 | *28* |

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

The extract and its isolated compounds exhibited significant antioxidant activity in DPPH assay. The $IC_{50}$ value of extract was observed to be 550µg/mL. The total antioxidant capacity of the extract and the isolated compounds were found to increase with increase in concentration. Among the isolated compounds phytol, Octonyl glucopyranose and hexonyl glucopyranose showed significant antioxidant activities with $IC_{50}$ values of 758, 482 and 645µg/mL respectively. The results from the table clearly indicate that the antioxidant activities of the Octonyl glucopyranose and hexonyl glucopyranose at the concentration of 800 µg/mL are almost same when compared to the chloroform extract. Based on the significant antioxidant potential, the phytol, Octonyl glucopranose and hexonyl glucopyranose were tested for cytotoxicity and compared with the chloroform extract.

## Cytotoxic activity by MTT assay

Carcinogenesis is a multistage process with an accumulation of genetic alterations resulting in the tumor. With the aim of preventing or slowing down or reversing the process of carcinogenesis, induction of apoptosis is considered to be one of the most important targets in a preventive approach [21]. To compare the cytotoxicity of phytol, Octonyl glucopyrnaose, hexonyl glucopyranose with combined chloroform extract, the MTT assay was carried out against HepG2 cells and the results are given in

Figure.2. The results indicate that the extract and Octonyl glucopyranose demonstrated marked cytotoxic activity on HepG2 cell type with an $IC_{50}$ value of 50 µg/mL and 93.71µg/mL. Among the three isolated compounds studied, Octonyl glucopyranose exhibited the most potent cytotoxic activity towards the HepG2 cells. In order to understand its mechanism, Octonyl glucopyranose was used for further investigations.

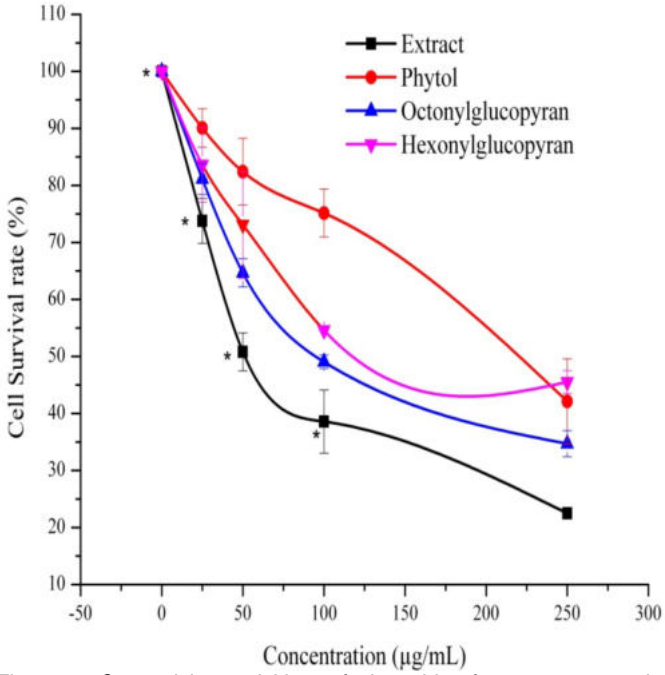



**Figure.2. Cytotoxicity activities of the chloroform extract and threeof its isolatedCompounds by MTT assay**

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

*Denotes that the values are significantly different (p<0.01) compared with control

## Activation Caspase-3 and Caspase-9 by Octonyl glucopyranose

Caspases present in mitochondria are the crucial mediators of apoptosis. To understand the mechanism, Octonyl glucopyranose was treated with caspase protein levels and the caspase-3 and caspase-9 were subjected to study. Among the 14 caspases identified in mammals, caspase-3 (previously called CPP32, Yama, apopain) is the major downstream protease in all apoptotic pathways [22-24]. As shown in figure. 3, the caspase-3 and caspase-9 levels are activated by the treatment with Octonyl glucopyranose in a dose dependent manner. To confirm the enhanced protein expression caused by the alteration of the mRNA level, the Octonyl glucopyranose was subjected to RT-PCR analysis.

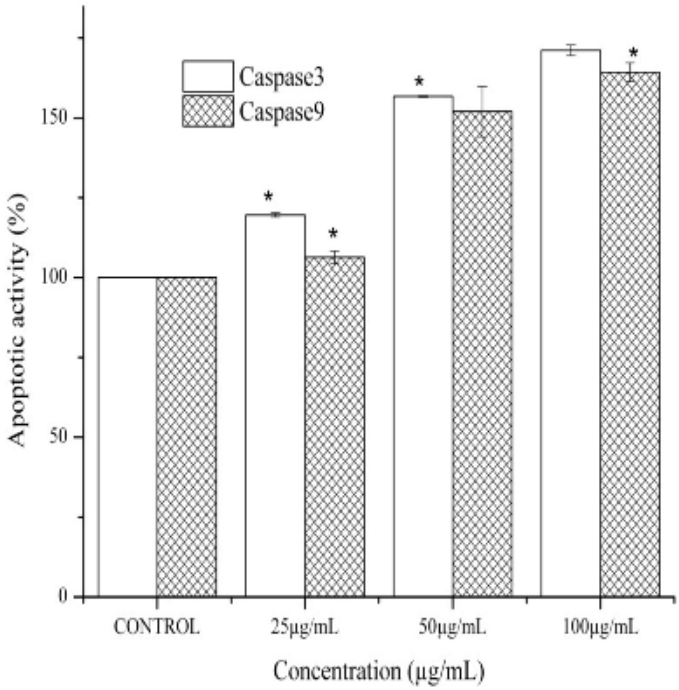



**Figure.3. Effect of Octonyl glucopyranose on Caspase-3 and Caspase-9**

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

*Denotes that the values are significantly different (p<0.01) compared with control

## Reverse transcriptase Polymerase Chain Reaction (RT-PCR)

In order to examine the cytotoxicity of Octonyl glucopyranose, it was related to the induction of apoptosis. The HepG2 cells were treated with Octonyl glucopyranose and were considered for RT-PCR studies. Full length cDNA was subjected to amplification using p53 primers and compared with the treated cell line (L4) with untreated cells line (L3) and the results are given in figure. 4. L1, L2 and L5 were 1kB ladder, positive and negative control of RT. The p53 gene was augmented in treated cells to high levels than the untreated cells and yielded a band size of 854bp. This is the first study to show that Octonyl glucopyranose induced p53 up-regulation in HepG2 cells and activation of Caspase-3 and -9 protein levels. The p53 plays an important role in the induction of apoptosis and it was feasible to induce p53 expression through administration of Octonylglucopyranose as a therapeutic strategy for preventing liver cancer.

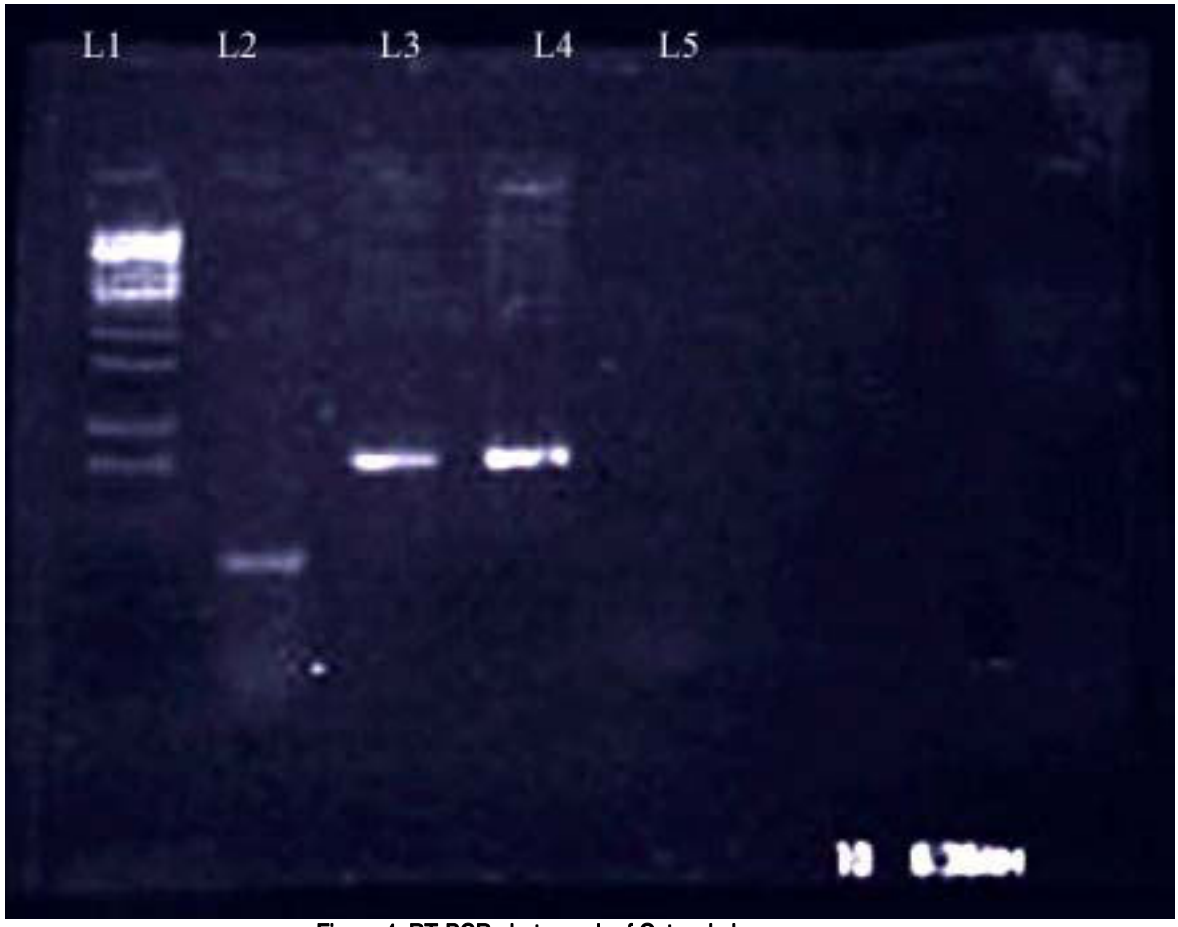



**Figure.4. RT-PCR photograph of Octonyl glucopyranose**

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

## Conclusions

The results of the study revealed that the *Morinda pubescens* possessed antioxidant and cytotoxic activities on HepG2 cell. The extract and the three of its isolated compounds phytol, Octonylglucopyranose and hexonyglucopyranose from *Morinda pubescens* leaf exhibited strong antioxidant and cytotoxic effect in a dose dependent manner. Octonylglucopyranose, one of the isolated compounds found to possess good anticancer activity and this could be a promising novel anti-cancer drug for liver cancer in near future.

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