Research Article

ISOLATION AND CHARACTERIZATION OF A NEW STEROIDAL DERIVATIVE AND EVALUATION OF ITS ANTIOXIDANT POTENTIAL FROM *DELONIX REGIA* (BOJ. EX. HOOK) RAF

M. Surendra Kumar[1]*, N. Astalakshmi[2], Aparna. P[3], Poojitha.K[3], Karishma. S. K[3] and Sivajyotsna. P[3].
1. Dept of Pharmacognosy, Devaki Amma Memorial College of pharmacy, Chelembra, Malapuram district. Kerala. India.
2. Dept of Pharmacy, Periyar College of Pharmaceutical Sciences, Thiruchirapalli. India.
3. Dept of Pharmacy/SCBT, SASTRA University, Thanjavur, India.
***Corresponding author's E-mail:** skshravansk@gmail.com

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ABSTRACT

In the current study, an attempt has been made to explore the phytoconstituents of *Delonix regia* (Boj. ex Hook) Raf through various phytochemical methods and to evaluate its antioxidant potential. Ethanolic extract of *Delonix regia* leaves is subjected to column chromatography using gradient elution technique. The petroleum ether and chloroform (1:1) fraction, which showed the presence of a single sterol is subjected to structural elucidation by spectroscopical methods such as UV, IR, ^{13}C NMR, ^{1}H NMR and mass spectroscopical studies. The isolated sterol is subjected to antioxidant studies by means of DPPH radical scavenging activity, nitric oxide radical scavenging assays and reducing power ability and lipid peroxidation assay. Ascorbic acid is used as the standard. The isolated compound is characterized as a cholestanol derivative, (16-(3,7,11,15-Tetramethyl-2-hexadecen-1-ol)-2-methylene(3α,5α)-cholestan-3-ol) and it is poor in its antioxidant potential as compared to the standard. *Delonix regia* (Boj. ex Hook) Raf which is known only for its ornamental value is found to be rich in its phytoconstituents. Isolation and characterization of phytoconstituents of the same may leads to the presence of a novel chemical entity which may be named as 16-(3,7,11,15-Tetramethyl-2-hexadecen-1-ol)-2-methylene(3α,5α)-cholestan-3-ol. However, the isolated sterol is found to be a very week antioxidant as compared to the standard.

Keywords: *Delonix regia*, phytoconstituents, 16-(3,7,11,15-Tetramethyl-2-hexadecen-1-ol)-2-methylene(3α,5α)-cholestan-3-ol and antioxidant.

INTRODUCTION

Delonix regia (Boj. ex Hook.) Raf is an ornamental tree and also useful shade tree in tropical conditions because of its dense foliage. It is a native of Madagascar with voluminous red and orange blooms. It is also well known as Gulmohar or Royal Poinciana. Various phytoconstituents are identified in seed, flower, bark, wood and leaves of drugs such as β-sitoserol, tannins, saponins, flavonoids, steroids, alkaloids and carotene hydrocarbons.[1]

Antioxidants are compounds that delay the oxidation of other molecules by inhibiting the initiation or propagation of oxidizing chain reactions. Recently, more attention was paid to the antioxidants because of increased concerns about oxidative stress in the human body.[2] Oxidative stress, induced by reactive oxygen species (e.g., O_2) and nitrogen (e.g., NO_2) species formed during normal metabolic processes, is believed to cause irreversible damage to cellular components such as lipids, proteins and DNA and can lead to various degenerative diseases. It has been reported that the natural antioxidants present in many plants reduce such damage and help to prevent mutagenesis, carcinogenesis and aging due to their radical scavenging activities.[3]

MATERIALS AND METHODS

Plant materials

Delonix regia (Boj. ex Hook) Raf leaves were collected from in and around areas of Thanjavur and the same was authenticated and a specimen copy of the same is deposited in department of CARISM, SASTRA University.

Extraction

Fresh leaves of *Delonix regia* (Boj. ex Hook) Raf were collected shade dried at room temperature, pulverized and extracted with 95% ethanol in a soxhlet extractor. The extract was concentrated in a rotary flash evaporator to yield a sticky residue of 25.85%w/w.

Isolation of phytoconstituents

The concentrated ethanolic fraction was fractioned by means of column chromatography using silica gel (60- 120 mesh size) with gradient elution technique. The fractions were collected and subjected to preliminary phytochemical and TLC studies. The petroleum ether–chloroform (1:1) fraction which shows the presence of a single phytoconstituent was selected and subjected for its identification, elucidation and evaluation.

Spectral studies

The isolated compound was subjected to the spectral studies such as UV, IR, 13CNMR, IHNMR and Mass spectroscopy. UV spectra were recorded on a JASCO V-530 spectrometer. The IR spectra were determined on a Shimadzu FT-IR 8000 spectrophotometer. Mass spectral studies were carried out using GCMS of Perkin Elmer make with Electron impact-mass spectroscopy (EI-MS) ionization techniques. NMR spectra were recorded on a Bruker 300MHz spectrometer for 1H and 13C,

respectively using deuteron chloroform (CDCl3) as the solvent. Chemical shifts were expressed in δ (ppm) down field from tetramethylsilane (TMS) as an internal standard and coupling constants reported in Hz.

Anti-oxidant activity studies

Oxidative stress caused by free radicals has become an area of interest in understanding the process of human diseases. The term oxidative stress has rarely been defined in a universal accepted way. One accepted definition by Sies in 1991 is, "a disturbance in pro-oxidant-anti-oxidant balance in the favour of the former, leading to the potential damage".[4-6]

The anti-oxidant activity of the isolated compound was studied by means of determining the total reducing power ability, nitric oxide radical scavenging assay, DPPH radical scavenging assay and assay of lipid peroxidation.

1. **Total reducing power ability**

The reducing power ability of isolated compound was investigated by the Fe^{3+}-Fe^{2+} transformation in the presence of extract. The reductive ability was measured by mixing 1ml of the sample prepared with distilled water to 2.5ml of phosphate buffer (0.2 m, pH 6.6) and 2.5 ml of 1% potassium ferri cyanide and incubated at 50^0C for 30 minutes. After that 2.5ml of trichloro acetic acid (10%) were added to the mixture and centrifuged for 10 minutes at 3000rpm, 2.5ml from the upper part were diluted with 2.5ml of water shaken with 0.5ml of fresh 0.1% ferric chloride. The absorbance was measured at 700nm using UV Spectrophotometer. The reference solution was prepared as above and contains water instead of the samples. Increased absorbance of the reaction mixture indicated increased reducing power. Vitamin C is used as the standard.[7-9]

2. **DPPH radical scavenging assay**

The free radical scavenging activity of isolated compound of *Delonix regia* (Boj. ex Hook) Raf. was measured *in vitro* by 1,1-diphenyl-2 picryl hydrazyl (DPPH) assay. The hydrogen donating ability of the compound was determined in the presence of DPPH stable radical. The isolated compounds and stock solutions (1mg/ml) was diluted to required concentrations in methanol. 1ml of 0.1mM DPPH methanol solution was added to 1ml of sample solution of different concentration and allowed to react at room temperature. After 30 minutes the absorbance was measured at 517 nm. The values obtained were converted into percentage anti oxidant activity (AA%) using the following formula[8,10-13]

$$AA\% = \frac{[(Abs_{control} - Abs_{sample}) \times 100]}{Abs_{control}}$$

Vitamin C (Ascorbic acid) was used as the standard. The IC_{50} values were calculated by linear regression of plots.

3. **Nitric oxide radical scavenging assay**

Nitric oxide generated from sodium nitroprusside in aqueous solution at physiological p^H interacts with oxygen to produce nitric ions, which were measured by griess reagent. The reaction mixture with a final volume of 3ml per tube containing 2ml of 10mM Sodium nitroprusside in phosphate buffer saline solution and was incubated with 1ml of different concentration of test compounds dissolved in phosphate buffer (0.025M, p^H 7.4) and the tubes were incubated at 25^oC for 150 minutes. Control experiments were carried out without the test compounds but with equivalent amount of buffer solution. There after 0.5ml of incubation solution was removed and diluted with 0.5ml of griess reagent (1% sulphanilamide in 2% O-phosphoric acid and 0.1% naphthalene diamine hydrochloride) and allowed to react for 30 minutes. The absorbance of the chromophore formed during diazotization of nitrite with sulphanilamide and subsequent coupling with naphthalene diamine dihydro chloride was read at 546 nm. The percentage inhibition was calculated using the formula.[8,14,15]

$$\text{Percentage Inhibition (I)} = \frac{(A_0 - A_1)}{A_0} \times 100$$

Where A_0 was the absorbance of the control reaction and A_1 was the absorbance of the test compound. The experiment was done using Vitamin C as standard.

4. **Estimation of TBAR's (Lipid Peroxidation Method)**

Thiobarbituric acid (TBA) reacts with malonyl dialdehyde (MDA) to form a diadduct, a pink chromogen, which can be detected spectrophotometrically at 532nm. The liver homogenates were prepared from normal male rats (250gms). The perfused liver was isolated and 10% w/v homogenate was prepared with homogenizer with 0.5M KCl. The homogenate was centrifuged at 800rpm for 15min and clear cell free supernatant was used for the study of *in vitro* lipid peroxidation. Various concentrations of the isolated compounds were dissolved in methanol and taken in test tubes. One ml of 0.5M KCl and 0.1ml of homogenates were added to the test tubes. Peroxidation was initiated by adding 100µl of 0.2mM ferric chloride. After incubation at 37°C for 30min, the reaction was stopped by adding 2ml of ice cold hydrochloric acid (0.25N) containing 15% trichloro acetic acid (TCA), 0.38% TBA and 0.5% BHT. The reaction mixture was heated at 80°C for 60min. The samples were cooled and centrifuged and the absorbance of supernatant was measured at 532nm.[16-18]

$$\text{Percentage inhibition (I)} = \frac{(A_0 - A_1)}{A_0} \times 100$$

Where A_0 is the absorbance of the control reaction and A1 is the absorbance of the test compound. The experiment was done using vitamin C as standard.

RESULTS AND DISCUSSIONS

Isolation of Phytoconstituents

In the present study, fractionation of the ethanolic extract of leaves of *Delonix regia* (Boj. ex Hook) Raf yield a phytoconstituent by means of column chromatographic technique. The isolated compound was subjected to spectral studies and further evaluated for its antioxidant potential.

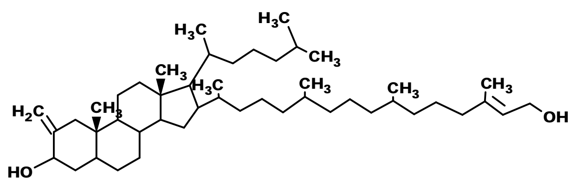
Characterization of the phytoconstituent

The UV absorbance spectra of the compound have shown a λmax at 275nm. FT-IR spectral studies of the compound showed a stretching at 3318cm^{-1} which corresponds to –OH stretching. Further stretching and deformations were seen at 2850cm^{-1}, 2728cm^{-1}, 1666cm^{-1}, 1600cm^{-1}, 1378cm^{-1}, 1281cm^{-1}, 1218cm^{-1}, 1037cm^{-1} and 836cm^{-1} corresponding to -CH stretching, -C=0 stretching in steroid ester, -C-C multiple bond stretching, -C-CH3 stretching, -C-H Deformation, -C-O stretching, Phenolic OH stretching, -C-C stretching and aromatic substitution respectively.

^{1}H NMR spectra of the isolated compound has shown signals at 7.6δ, 5.4δ, 4.2δ, 2.0δ, 1.7δ and 1.3δ indicating the occurrence of an aryl proton, aromatic OH proton m Alkyl OH proton, -CH-C=O proton , Non conjugate proton and methyl proton of the compound. The ^{13}CNMR spectral studies of the compound showed a signal 162δ for the carbonyl group attached to aromatic ring followed by –CH_2. The presence of an aromatic ring was confirmed by the exhibition of a signal at 130δ. There was a signal observed at 129δ indicating the occurrence of a double bond with the aromatic nature. Further there were signals at 032δ, 31.6δ, 028δ, 24.3δ and 24.1δ indicating C-8, C-2, C-25, C-15 and C-22 groups. The signal at 022δ indicates the possibility of C-26 carbon attachment to any group. The methyl group carbon C-21 was confirmed by means of occurrence of a signal at 018δ. There were 2 signals at 12δ and 19.4δ indicating C_{18} and C_{19} in sterol ring.

The EI-MS spectrum of the isolated compound revealed significant fragment ions at m/z 268[$C_{18}H_{36}O$]$^+$, 400[$C_{28}H_{48}O$]$^+$, 296[$C_{20}H_{40}O$]$^+$, 278[$C_{18}H_{36}O$]$^+$ and [$C_{30}H_{50}O$]$^+$. This fragmentation indicates the isolated compound must be of Cholestan-3-ol.

Based on the spectral studies, the isolated compound was found to be a cholestanol derivative and it may be named as 16-(3,7,11,15-Tetramethyl -2-hexadecen-1-ol)-2-methylene(3α,5α)-cholestan-3-ol.



16-(3,7,11,15-Tetramethyl-2-hexadecen-1-ol)-2-methylene(3α,5α)-cholestan-3-ol.

Antioxidant studies of 16- (3, 7, 11, 15-Tetramethyl -2-hexadecen-1-ol)-2-methylene(3α,5α)-cholestan-3-ol

The isolated compound (16-(3,7,11,15-Tetramethyl-2-hexadecen-1-ol)-2-methylene(3α,5α)-cholestan-3-ol) was subjected for evaluation of its antioxidant potential by means of total reducing power, DPPH radical scavenging assay, nitric oxide radical scavenging assay and lipid peroxidation assay using Vitamin C as the standard reference drug.

IC_{50} values of the isolated compound and the standard was determined and found to be 3.1μg/ml, 2.7 μg/ml and 60 μg/ml for DPPH, Nitric oxide and Lipid peroxidation assay of the standard drug ascorbic acid. However, the isolated compound (16-(3,7,11,15-Tetramethyl-2-hexadecen-1-ol)-2-methylene(3α,5α)-cholestan-3-ol) was found to be a very week antioxidant and its IC_{50} values were non comparable to that of the standard.

Discussion

In the present study *Delonix regia* (Boj. ex Hook) Raf, which is known only for its ornamental purpose was explored for its phytoconstituents resources. The ethanolic extract of the leaves of *Delonix regia* (Boj. ex Hook) Raf was subjected to column chromatography by gradient elution technique using solvents ranging from non polar to polar in various combinations. The petroleum ether–chloroform (1:1) fraction yielded a single compound with melting point 142°C is subjected for preliminary phytochemical evaluation followed by TLC studies, confirmed its occurrence as a steroidal compound. This compound was subjected to spectral studies such as UV, IR, NMR and Mass spectroscopy. The spectral studies also confirmed the occurrence of steroidal derivative in the fraction of the ethanolic extract. Based on the spectral data's obtained the isolated compound can be named as 16- (3, 7, 11, 15-Tetramethyl -2- hexadecen-1-ol)-2-methylene(3α,5α)-cholestan-3-ol.

Assay of reducing activity was based on the reduction of Fe3+/ferricyanide complex to the ferrous form in presence of reductants (antioxidants) in the tested samples. The Fe2+ was then monitored by measuring the formation of Perl's Prussian blue at 700 nm.[9] The reducing power of the isolated compound increased with the concentration. However, this increase was not found to be as comparable to that of the standard ascorbic acid suggesting that the isolated compound 16- (3, 7, 11, 15-Tetramethyl -2- hexadecen-1-ol)-2-methylene(3α,5α)-cholestan-3-ol may be a poor antioxidant.

The free radical scavenging activity of the isolated compound 16- (3, 7, 11, 15-Tetramethyl -2- hexadecen-1-ol)-2-methylene(3α,5α)-cholestan-3-ol from *Delonix regia* leaves was evaluated through DPPH method and compared with the standard drug ascorbic acid. DPPH radical scavenging was found to be one of the most frequently used method for evaluation of the antioxidant capacity of the compounds or extracts and it has been

generally used as a means of quickly evaluating the antioxidant activities of specific compounds or extracts.[19] In the DPPH method, the antioxidants react with the stable free radical i.e.,α,α -diphenyl-b-picrylhydrazyl (deep violet colour) and convert it to α,α -diphenylb-picrylhydrazine with discolouration. The discolouration indicates the scavenging potentials of the sample antioxidant such as phenolic compounds. DPPH was a free radical which accepts an electron or hydrogen radical to become a stable diamagnetic molecule. [20] The reduction capability of DPPH radical was determined by the decrease of absorbance induced by plant antioxidants. The isolated compound was found to be a poor antioxidant as compared to the standard drug.

Nitric oxide - free radical which had some important physiological role, was less reactive. But its metabolic product – peroxynitrite – formed after reacting with O_2 was extremely reactive and directly induce toxic reaction including SH group oxidation, protein tyrosine nitration, lipid peroxidation and DNA modification.[21] Antioxidant drug should have the capability to scavenge the nitric oxide radical. Isolated 16-(3, 7, 11, 15-Tetramethyl -2-hexadecen-1-ol)-2-methylene(3α,5α)-cholestan-3-ol was found to be a very weak antioxidant by exhibiting non significant nitric oxide radical scavenging potential as compared to the ascorbic acid.

The TBA test measures the amount of malondialdehyde (MDA) produced after the decomposition of the lipid peroxide during the oxidation process. At a low pH and high temperature (100°C), MDA binds TBA to form a pink complex that can be measured at 532 nm. The isolated derivative shows rapid increase in percentage inhibition with respect to the increase in concentration. However, the results were highly insignificant as compared to the standard drug.

These results suggested that 16- (3, 7, 11, 15-Tetramethyl -2- hexadecen-1-ol)-2-methylene(3α,5α)-cholestan-3-ol, was found to possess antioxidant property but not as significant as that of the standard drug. Further purification of the same may leads it to a novel phytoconstituent.

CONCLUSION

The present study of *Delonix regia* (Boj.ex Hook) Raf aims at exploring the phytoconstituents of it and to characterize them along with their antioxidant potential. Isolation of a compound was done and maximum possibility of characterization of isolated compounds was carried out. The isolated compound may be found to be as (16-(3,7,11,15-Tetramethyl-2- hexadecen-1-ol)-2-methylene (3α,5α)-cholestan-3-ol). The isolated cholestanol derivative was evaluated for its antioxidant activity using ascorbic acid as standard and the results were found to be nonsignificant. The isolated cholestanol derivative showed very poor antioxidant activity. It can be concluded that the *Delonix regia* (Boj.ex Hook) Raf, which is known only for its ornamental properties can be explored for its novel phytoconstituents and tried out for various pharmacological actions in future.

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