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Original article

Effect of two andrographolide derivatives on cellular and rodent models of non-alcoholic fatty liver disease

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ABSTRACT

The prevalence of Non-Alcoholic Fatty Liver Disease (NAFLD) is increasing and there is an increasing interest in natural products to treat NAFLD. This study aimed to evaluate the hepatoprotective effect of andrographolide and two of its derivatives; in one the OH group at C-14 was removed and in the other OH groups at C-3 and C-19 were protected. Andrographolide (AN) was isolated from the aerial parts of *Andrographis paniculata* Wall. Isoandrographolide (IAN) and 3,19-acetonylidene andrographolide (ANA) were derivatized from AN. Drug likeness of the compounds was studied using DataWarrior. The effect of the compounds in ameliorating hepatic steatosis and lipotoxicity was assessed using palmitate-oleate induced steatotic HepG2 cell lines. *In vivo* efficacy of the compounds was assessed by using HFD fed rats. IAN showed comparatively high drug score and low irritability than AN. MTT assay indicated that the treatment with IAN had comparatively less toxicity than AN and ANA to HepG2 cells. The treatment with IAN significantly reduced the lipid accumulation and the leakage of LDH and transaminases, while the treatments with AN and ANA did not prohibit the leakage. In the *in vivo* experiment, the treatment with IAN showed comparatively better hepatoprotection by reducing the serum lipid, transaminases and ALP levels than with AN and ANA. Our results showed that IAN could be a promising lead to treat NAFLD with comparatively low toxicity and improved efficacy.

1. Introduction

Non-Alcoholic Fatty Liver Disease (NAFLD) is one of the most common liver diseases; its prevalence is increasing parallelly with metabolic syndromes. It is a progressive liver disease leading to fibrosis, cirrhosis and hepatocellular carcinoma in the absence of significant alcohol consumption [1]. The global prevalence of NAFLD was said to be 25.24% [2] and an analysis indicated that in India about at least 25 million patients with NAFLD are having risk for significant liver disease [3]. Lifestyle changes were shown to improve the histology in the liver of NAFLD subjects [4,5]. However, therapeutic options to treat NAFLD or Non-Alcoholic Steato-Hepatitis (NASH) are only limited and medications with long term efficacy that beneficially affect fibrosis are lacking [6]. There is an increasing interest to identify more natural products such as Siberian ginseng, green tea, turmeric etc., to prevent/treat NAFLD [7].

Andrographis paniculata (Burm.f.) Wall. ex. Nees (Acanthaceae) is one of the important medicinal plants used in the treatment of various illnesses including liver disorders in traditional Indian system of medicine [8]. A small, randomized, controlled trial with 60 hypertriglyceridemic subjects showed that the administration of *A. paniculata* extract significantly lowered the TG levels [9]. Various preclinical experiments reported the anti-inflammatory and hepatoprotective effect of *A. paniculata* and its major constituent, andrographolide (AN), a labdane diterpenoid [10,11]. The treatment with AN reduced the high fat diet induced obesity and steatosis in HFD fed mice through the

Abbreviations: ALP, Alkaline Phosphatase; ALT, Alanine Transaminase; AN, Andrographolide; ANA, 3,19 Acetonylidene Andrographolide; ANOVA, Analysis of Variance; AST, Aspartate Transaminase; BSA, Bovine Serum Albumin; b.w., Body weight; $CDCl_3$, deuterated chloroform; CMC, Carboxymethylcellulose; Conc. HCl, Concentrated Hydrochloric acid; COX-2, Cyclooxygenase-2; DMEM, Dulbecco's Minimal Essential Medium; DMSO-d_6, deuterated dimethyl sulfoxide; FBS, Fetal Bovine Serum; FT-IR, Fourier Transform Infra-red Spectroscopy; GI_{25}, Growth Inhibition Concentration$_{25}$; HFD, High Fat Diet; IAN, Isoandrographolide; KBr, Potassium Bromide; LDH, Lactate Dehydrogenase; MNC, Mono-Nuclear Cells; MTT, [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide]; NaCl, Sodium Chloride; NAFLD, Non-Alcoholic Fatty Liver Disease; NASH, Non-Alcoholic Steato-Hepatitis; NFD, Normal Fat Diet; OH, Hydroxyl; ORO, Oil Red - O; PBS, Phosphate Buffered Saline; PO-BSA, Palmitate, oleate, BSA complex; SD, Standard Deviation; T2D, Type 2 Diabetes; TC, Total Cholesterol; TG, Triglycerides

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suppression of Sterol Regulatory Element Binding Protein pathway [12]. Many semisynthetic derivatives were prepared from AN with improved bio-efficacies for various diseases [13,14]. Some semisynthetic derivatives of AN were also tried to improve antihyperlipidemic [15,16] and hepatoprotective [17] effects.

The three OH groups (C-3, C-14 and C-19), two double bonds ($\Delta^{12(13)}$ and $\Delta^{8(17)}$) and one α-alkylidene-γ-butyrolactone group, are the main targets for structural modifications in AN [18]. Among them, the OH groups are the most common sites of modifications and previous studies indicated that the OH at C-14 was involved with the cytotoxicity of AN [14]. A study reported that the protection of hydroxyl groups at C-3 and C-19 improved anti-inflammatory efficacy of AN [18,19] and no structure activity relationships were established for the hepatoprotection and anti-NAFLD effects of AN [17]. We have evaluated the anti-NAFLD effect of two AN derivatives, viz., isoandrographolide (IAN), which lacked OH at C-14 and 3,19 acetonylidene andrographolide (ANA) where OH at C-3 and C-19 was protected, using *in vitro* and *in vivo* models of NAFLD.

2. Materials and methods

2.1. Phytochemistry

2.1.1. Chemicals and instrumentation (Chemistry)

Solvents and reagents of analytical grade were purchased from Qualigens and used without further purification unless otherwise indicated. All the reactions were done in oven-dried glasswares. FT-IR spectrum was recorded on a Perkin-Elmer FT-IR grating spectrophotometer (Spectrum Two) in KBr disc. ^{1}H and ^{13}C NMR spectra were recorded on Bruker AV-500 (500 and 125 MHz) with $CDCl_3$ and DMSO-d_6 as the solvents. Chemical shifts were recorded as δ values in parts per million (ppm) with TMS as the internal standard.

2.1.2. Plant material

Aerial parts of *A. paniculata* were procured from local market of Chennai and authenticated by one of the authors (PP) of this communication. Voucher specimen (ERI-EP-ET-04) of the crude drug was deposited in the herbarium of Entomology Research Institute, Loyola College, Chennai for future reference.

2.1.3. Isolation of andrographolide (AN)

The plant materials were shade dried and coarsely powdered. The powder (3 kg) was soaked in 7.5 L methanol for 48 h by cold percolation method, under continuous stirring. The contents were filtered and concentrated under reduced pressure at 45 °C to 1.5 L. Concentrated filtrate was then refluxed with activated charcoal (30 g) for 15 min and filtered. The filtrate was concentrated and kept at 4 °C to yield crude andrographolide. Repeated crystallizations with methanol yielded pure AN (yield: 12.5 g; purity: 99.5%) [20–22].

2.1.4. Preparation of Isoandrographolide (IAN)

Isoandrographolide was prepared according to previously published procedure [15]. To 5.0 g of AN, 81 mL of Conc. HCl was added and the mixture was stirred at room temperature for 24 h. The whole solution was poured into ice cold water (200 mL) and extracted with dicholoromethane (2 × 200 mL). The combined organic layer was washed twice with water, dried over anhydrous Na_2SO_4; crystallization with ethyl acetate yielded IAN (yield: 3.2 g; purity: 98.5%).

2.1.5. Preparation of 3,19 Acetonylidene Andrographolide (ANA)

Acetonide production was done on the basis of previously published protocol [23]. To 5.0 g of AN dissolved in 5 mL of DMSO, 70 mL of 2, 2-dimethoxy propane and 2.0 g pyridinium-*p*-toluene sulfonate were added. The mixture was stirred at room temperature for 30 min. The mixture was poured into ice-cold water and extracted with dichloromethane (2 × 200 mL). The combined organic layers were washed with water, dried over anhydrous Na_2SO_4 and concentrated. Crystallization with methanol yielded ANA (yield: 3.4 g; purity: 99.2%).

2.1.6. In silico studies

The data regarding partition coefficient (clog *P*), counts of hydrogen bond acceptors and donors, mutagenicity, tumorogenecity, reproductive effect, irritability and drug likeness of AN and its derivatives were predicted using DataWarrior software (Version 4.5.2) [24]. Drug score was also calculated based on the above values using the same software.

2.2. Evaluation of in vitro anti-NAFLD effect of AN, IAN and ANA using HepG2 cell lines

2.2.1. Chemicals (Bioassays)

Sodium oleate, Sodium palmitate and Fenofibrate were purchased from Sigma Aldrich; DMEM and FBS were purchased from Gibco. Antibiotic-antimycotic mixture, trypsin, DMSO, ORO, MTT dye, LDH assay kit and all other chemicals of analytical grade were obtained from Hi-Media chemicals. TG estimation and protein estimation kits used for *in vitro* assays were purchased from Merck and Bio-Rad, respectively. All other kits used for biochemical estimations were purchased either from Agappe diagnositics or from Accurex biomedicals.

2.2.2. Cell culture

HepG2 cells were obtained from National Center for Cell Sciences, Pune (India) and cultured in 5% CO_2 incubator at 37 °C using DMEM supplemented with FBS (10%), penicillin (100 U/mL) and streptomycin (100 μg/mL). The cells were sub-cultured at 75% confluence by total media replacement using 0.25% (w/v) trypsin − 0.53 mM EDTA every 2–3 days. DMSO was used as the vehicle and its level was set as 0.01% for all the *in vitro* bioassays.

2.2.3. Assessing the toxicity of AN, IAN and ANA on HepG2 cell lines using MTT assay

The cytotoxic effect of the AN and its derivatives were analyzed by using MTT assay as described by Mosmann with minor modification [25]. HepG2 cells were seeded at 5×10^3 cells/well into a 96-well plate and incubated for 24 h. Spent media were then replaced with fresh media containing AN, IAN or ANA (100–500 μM) dissolved in DMSO. Vehicle control cells were treated with same amount of DMSO alone. The plates were incubated in CO_2 incubator for 24 h. Then the spent media were replaced with sterile MTT solution (5 mg/mL in PBS at pH − 7.4; 100 μL/well) and incubated for another 3 h. The unreacted dye was removed, the formazan crystals were dissolved with DMSO (200 μL/well) and kept in dark for 30 min. The resulting purple colour was quantified by measuring absorbance at 570 nm. The assays were performed thrice and the results were expressed in percentage in comparison to vehicle treated cells. The following formula was used for calculation.

$$\text{Cell population growth percentage} = \left(\frac{A570(\text{testmaterial})}{A570(\text{control})}\right) \times 100$$

GI_{25} values were calculated using linear regression analysis and the dose ranges for further studies were kept below their GI_{25} values.

2.2.4. Induction of steatosis in HepG2 cell lines using PO-BSA mixture

Induction of steatosis was done in accordance with the protocol of Hetherington and coworkers with slight modification [26]. The final concentration of the fatty acids was kept as 1 mM which reflected the pathological circulating fatty acid levels in NAFLD [27]. Sodium palmitate (6 mM) and sodium oleate (4 mM) were taken at 2:3 ratio to achieve a final concentration of 10 mM and dissolved in PBS at 70 °C. This palmitate-oleate mixture was then complexed with BSA at a molar

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