

Original Research Article

Anti-hepatitis B viral activity of *Phyllanthus niruri* L (Phyllanthaceae) in HepG2/C3A and SK-HEP-1 cells

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Abstract

Purpose: To investigate the effectiveness of an ethanol extract of *Phyllanthus niruri* against hepatitis B viral (HBV) infection in human HepG2/C3A cells.

Methods: An ellagic acid-rich ethanol fraction was obtained from *P. niruri* (Euphorbiaceae) by extraction and thin-layer chromatography. The anti-HBV activity of the fraction was evaluated *in vitro* against HepG2/C3A cells. The physicochemical characteristics of the fraction were assessed by nuclear magnetic resonance (^1H and ^{13}C -NMR).

Results: The isolated active compound showed a half-maximal inhibitory concentration (IC_{50}) of 120 $\mu\text{g}/\text{mL}$. Ellagic acid had no effect on HBV DNA replication at the concentrations evaluated, and did not inhibit the reproduction of HBV. However, the ethanol fraction inhibited the growth of HBV-infected HepG2/C3A cells.

Conclusion: The findings suggest that the ethanol fraction of *P. niruri* inhibits HBV, and that the active component is not ellagic acid.

Keywords: *Phyllanthus niruri*, Anti-HBeAg, Hepatitis B virus

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INTRODUCTION

Hepatitis B virus (HBV) is a hepatotropic virus and the causative agent of hepatitis B. HBV infection may lead to hepatocellular carcinoma (HCC) and cirrhosis [1]. HBV infection may be symptomless in many people during the initial stages. Globally, HBV is responsible for 0.5–1 million deaths annually. Although a considerable portion of carriers have varied level of hepatocyte obliteration, the infection can lead to HCC or cirrhosis [2]. More than 300 million individuals are infected with HBV, which is thus considered a pandemic [3]. Treatments for HBV infection include standard and pegylated interferon-alpha and nucleoside analogues such as entecavir, lamivudine and adefovir. However, current therapies are unable to eradicate HBV.

Development of drug resistance and reactivation of viral replication can occur even when treatment is successful [4]. The aim of therapies for HBeAg-positive and -negative individuals should be long-term eradication of HBV DNA, with an endpoint of hepatitis B surface antigen (HBsAg) seroconversion [5].

Phyllanthus niruri L. (Euphorbiaceae) is a medicinal herb distributed in South Asia. In traditional medicine, the plant has long been used as a hepatoprotectant and for treatment of hepatitis B [6]. Current HBV therapies can result in moderate-to-severe side effects, development of resistance to HBV expansion, or limited efficacy in only a subset of HBV-infected patients [7]. Despite the availability of numerous antiviral drugs, a number of issues remain, such as

limited efficacy, dose-dependent side-effects, and drug resistance. Therefore, more-effective anti-HBV drugs are urgently needed. Despite their potential, few natural herbs have been evaluated in a systematic manner. The goals of HBV treatment are to prevent development of HCC and cirrhosis, regulate alkaline transaminase (ALT) levels, suppress viral replication, and reduce the liver damage in HBeAg-negative as well as with HBeAg-positive individuals [8].

It has been reported that water extract of *P. niruri* inhibits cellular DNA polymerase activity during HBV replication [9]. A considerable proportion of untreated newborns of mothers who are long-term HBeAg carriers become infected, and 90 % become long-term carriers [10]. The secretory non-particulate form of HBeAg is not necessary for HBV replication or infection. The immune system responds to the presence of HBeAg and suppresses infection [11].

The plant *Phyllanthus niruri* (also known as *Phyllanthus amarus*) exhibits anti-HBV activity and has long been used to treat jaundice in southern India [12]. *P. niruri* belongs to the family Euphorbiaceae and can be used to treat kidney stones [13] and disorders of the gastrointestinal and genitourinary tracts [14].

In this study, we isolated and characterized ellagic acid from *P. niruri* and assessed its effect on HBV-infected HepG2/C3A cells as a potential agent to prevent the development and progression of hepatitis virus and HCC in chronic HBV carriers [15,16].

EXPERIMENTAL

Evaluation of physicochemical properties

Cary 60 UV-Vis and 660 FTIR (together with potassium bromide) spectrophotometers (both from Agilent Technologies (Santa Clara, California, United States) were used to monitor UV-visible and infrared spectra, respectively. The acquired spectra were subjected to high-throughput mass spectrometry (electrospray ionization MS).

Bruker nuclear magnetic resonance (NMR) spectroscopy at 400 MHz, utilized to obtain the ^1H and ^{13}C NMR spectra in CDCl_3 . Electrothermal's melting point apparatus (Bibby Scientific Limited, Staffordshire, UK) was utilized to assess the melting point. Thin-layer chromatography was conducted on silica gel 60 F254 plates (250 μm).

Isolation and purification of an anti-HBV compound from *P. niruri*

Five hundred grams of dried *P. niruri* plant mass were subjected to aqueous extraction at 37 °C for 3 h. The extract was filtrated twice under low pressure at 40 °C (Figure 1). The supernatant was resolved on a gel column using a $\text{CHCl}_3/\text{MeOH}$ gradient twice. Fraction #12 was then separated on a silica gel column (70/10/1 $\text{CHCl}_3/\text{MeOH}/\text{H}_2\text{O}$) at low pressure. The resulting brown-colored powder was subjected to preparative HPLC using a mobile phase of acetonitrile/1 % formic acid (10/90 to 45/55 % gradients). Anti-HBV activity was evaluated as an increase in antigen secretion by HepG2/C3A cells (Figure 2). Extracellular HBeAg was detected using an ELISA kit.

Anti-HBV activity assay

HepG2/C3A cells were plated on 24-well flat-bottom culture plates at $3 \times 10^5/\text{mL}$. Following incubation for 24 h, test compounds were applied to the plates.

The compounds were dissolved in dimethyl sulfoxide (DMSO) to 1, 2, 4, 8, 16, 32, 64 and 128 μg . The highest concentration was 2.5 $\mu\text{g}/\text{mL}$, which did not affect HepG2/C3A cells. HBsAg levels were evaluated by enzyme immunoassay (Sigma-Aldrich Shanghai Trading Co Ltd, Shanghai, China). The reactions were assayed at a wavelength of 492 nm using the CLARIOstar® High-Performance Multimode Microplate Reader. Inhibition (%) was evaluated in comparison with DMSO as in Eq 1:

$$\text{Reduction (\%)} = \{(1 - \text{As}/\text{Ab})\}100$$

where As and Ab are the absorbance of test sample and blank, respectively.

Data are presented as the means of three replicates. Activity was classified as follows: 20 – 35 % as weak, 30 – 50 % as medium, 50 – 65 % as strong, and > 65 % as very strong. Cell damage was assessed by aspartate transaminase (AST) assay (Abnova Corporation, Taipei, Taiwan). AST values of > 25 IU/L were considered indicative of cell damage.

HBeAg assay

For the initial assessment of HBV reproduction, secreted levels of HBeAg from HepG2/C3A cells following treatment of *P. niruri* extracts or ellagic acid were evaluated by ELISA (Hepatitis B Antigen ELISA kit; DRG International Inc., Springfield, NJ, USA).

Assessment of HBV DNA levels

Cells were lysed in 0.5 mL lysis buffer (2 % sarkosyl, 7 % 2-mercaptoethanol, 4 M guanidine isothiocyanate) and then washed for 1 h in 1 mM EDTA, 50 mM Tris (pH 8.0) using a micro-dialysis device (Life Technologies, Gaithersburg, MD, USA). Next, Proteinase K was added, removed using chloroform and phenol and resuspended in ethanol as described previously [17,18]. Cells cultured in 10-cm-diameter dishes were disrupted in degenerative buffer (6 mL), and DNA was extracted as described elsewhere [25]. DNA (10 µg/lane) was digested by EcoRI, separated by electrophoresis in 1 % agarose gels, and transferred to nitrocellulose membranes. DNA fragments were radiolabeled with [³²P] dCTP 32P dCTP, using a nick-translation kit (Invitrogen, Carlsbad, CA, USA) as per manufacturer's instructions, and then hybridized with a specific probe (specific activity 3–5 × 10⁸ dpm/µg). Post-washing and hybridization were performed as described previously [9].

HBV polymerase assay

The medium from propagating HepG2/C3A cells was collected and treated with polyethylene glycol 8000 (PEG 8000) to precipitate nucleocapsids. Nucleocapsids were isolated from HBV-infected cells and assayed for endogenous polymerase activity. HBV polymerase activity was monitored by measuring the incorporation of [^{α-32}P]-labeled deoxynucleotide triphosphate (dNTP) into acid-precipitated products. Each 40 µL assay reaction consisted of 100 mmol/L Tris (pH 7.5), 10 mmol/L MgCl₂, 0.6 U/µL RNasin, 5 % glycerol, 0.2 µg/µL activated calf thymus DNA, 100 µmol/L unlabeled dNTPs, [^{α-32}P]-labeled dNTP (approximately 100 Ci/mmol), and ellagic acid at various concentrations.

To determine the inhibition constants for penciclovir triphosphate (dGTP substrate analog), adefovir diphosphate (dATP substrate analog), and lamivudine triphosphate (dCTP substrate analog), [^{α-32}P]-labeled dGTP, [^{α-32}P]-labeled dATP, and [^{α-32}P]-labeled dCTP were used, respectively. Five microliters of HBV polymerase (~ 0.1 µg) were added to initiate the reaction. Twelve-microliter aliquots were removed at 0–20 minutes and transferred onto Whatman 3MM paper discs. The paper discs were washed three times in 5 % trichloroacetic acid plus 1 % sodium pyrophosphate and once in 95 % ethanol.

The incorporated radioactivity was measured using liquid scintillation counting (LSC) (Amersham Bioscience, Uppsala, Sweden).

Computation of half-maximal inhibitory concentrations (IC₅₀)

Intracellular encapsidated HBV DNA was extracted and subjected to gel blot analysis. The IC₅₀ of each drug was determined as the concentration resulting in a 50 % decrease in the viral DNA level compared with that of untreated cells.

Statistical analysis

Data are shown as means ± standard deviation (SD, n = 3). IC₅₀ values were computed using Origin Lab version 18.0 software (Origin Lab Inc., Guangzhou, China). Students' non-paired t-test was used to compare factors between the sample and solvent control. A value of *P* < 0.005 was considered to indicate significance.

RESULTS

Anti-HBV activity of extract fractions

Figure 1 shows the antiviral activity of the various fractions of the plant extract.

Attributes of hepatitis B-reducing elements

The physicochemical properties of components with anti-HBV activity are shown in Table 1. Crystals of the active component were yellow in color. The component had a molecular weight of 302 Da, and infrared spectroscopy showed absorption at 3380, 1720, 1690, and 1610 cm⁻¹, indicating the presence of phenolic hydroxyl, α-byron C=O, and benzenoid C=C groups. These results together with other findings (melting point, UV spectra, R_f, and solubility) indicated that the active component is ellagic acid, C₁₄H₆O₈. The ¹H NMR spectrum of ellagic acid has a singlet at δ 7.47, which was associated with two protons at C-5 and C-5 (Table 2). The ¹³C NMR spectrum of ellagic acid exhibited quinone carbonyl carbons at 168.60 and 145.86, showing C-2+C-2, C-4+C-4, and C-3+C-3, which were associated with δ 157.58 and δ 149.01. In addition, δ 119.73 and 121.79 were assigned to four carbons at C-5, C-5 and C-1, C-1, and δ 117.3 was assigned to C-6, C-6. The ¹³C NMR data thus supported identification of the active component as ellagic acid.

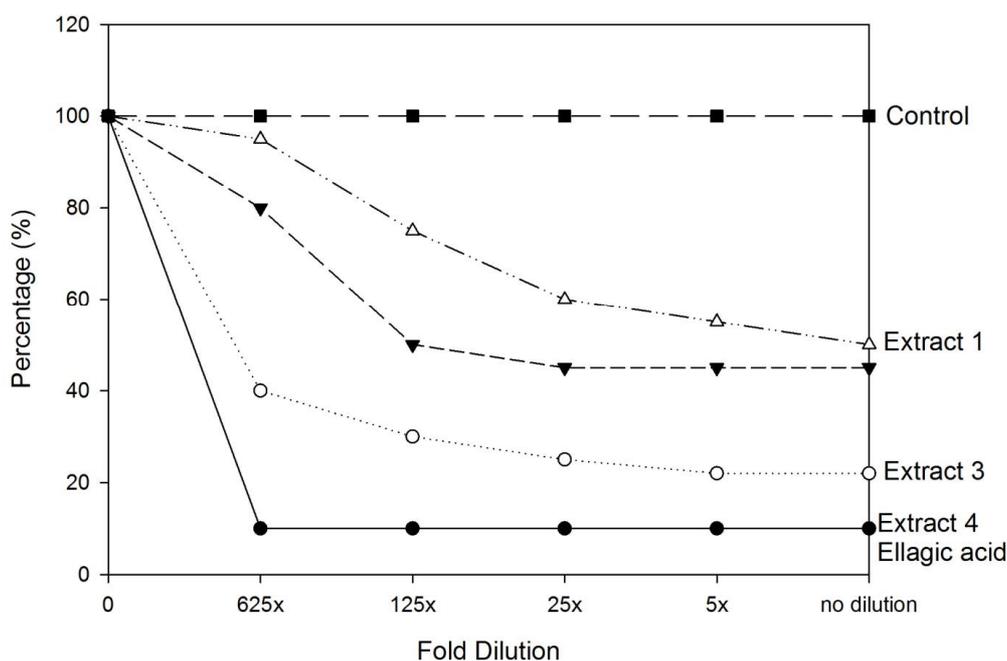


Figure 2: Anti-HBV activity of extract fractions

Table 1: Physicochemical properties of ellagic acid

Appearance	Yellow powder
Melting point (°C)	300–360
Molecular formula	C ₁₄ H ₆ O ₈
FAM-MS (m/z)	301 [M–H] [–]
UV, λ _{max} (nm)	300
IR, ν _{max} (nm)	3380, 1720, 1690 and 1610
R _f value (TLC)	0.67
Solubility	DMSO, ethanol and methanol

Effect of ellagic acid on HBeAg secretion

Fraction #12 exhibited dose-dependent toxicity towards HepG2/C3A cells (Figures 3 and 4). Ellagic acid was incapable of increasing HBV polymerase activity. PEG 8000 precipitated virion particles with four deoxyribonucleic acid was used to observe anti-polymerase functions of ellagic acid. Lamivudine 3TC triphosphate was used as the positive control (Figure 5).

We assessed whether the ellagic acid from *P. niruri* extract is responsible for the reduction in HBV polymerase activity. Ellagic acid at 10, 20, 40, and 60 µg/mL did not inhibit HBV replication (lanes 5–8) (Figure 6), unlike the positive control, 3TC (0.1, 1.0, and 10.0 mM) (lanes 2–4). Furthermore, 0.2 mM 3TC inhibited HBV replication in HepG2/C3A cells.

DISCUSSION

Chronic HBV infection can lead to development of cirrhosis and HCC, which increases the risk of death. The FDA has approved two drugs to treat HBV infection; however, both show poor efficacy and various side effects [20,21].

Medicinal plants have long been used to treat various illnesses worldwide. HBV infects millions of people worldwide and causes a severe, transmittable disease of the liver. The pharmaceutical industry is endeavoring to discover a natural anti-HBV agent. Therefore, it is important to explore phytochemicals as a new source of drugs. However, data on the antiviral activity of medicinal plants are limited. *Phyllanthus* species have long been used as herbal remedies for HBV infection.

Table 2: ¹H and ¹³C NMR spectral data for ellagic acid in DMSO-d₆

Position	¹³ C chemical shift	¹ H chemical shift
1	121.790	-
2	145.867	-
3	149.015	-
4	157.58	-
5	119.734	7.47(s)
6	-	7.47(s)
7	117.161	-
8	168.601	-

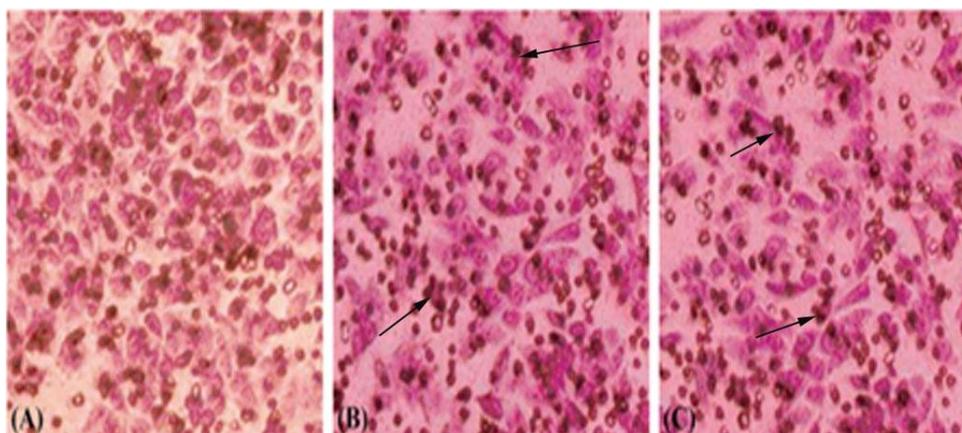


Figure 3: Effect of ellagic acid on HepG2/C3A cells. (A) Control and (B) 64 and (C) 128 µg/mL ellagic acid

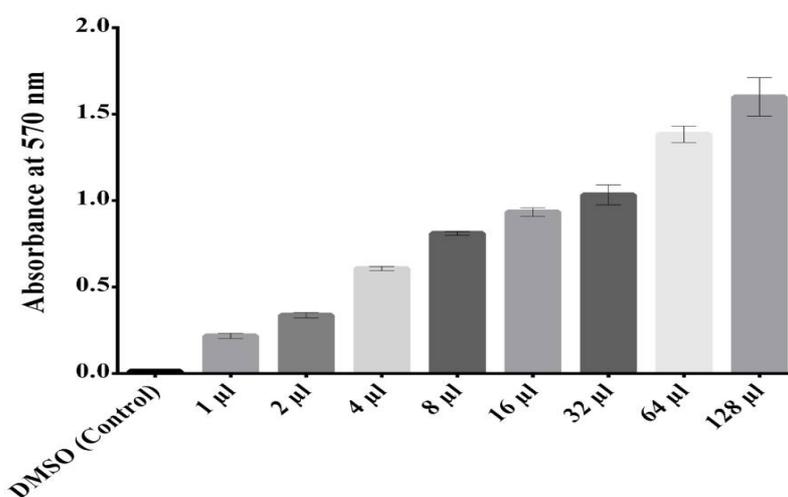


Figure 4: Dose-dependent toxicity of ellagic acid

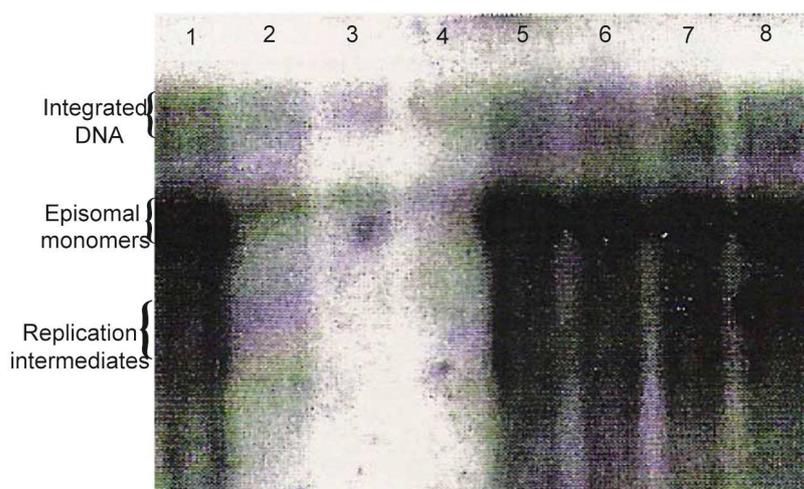


Figure 5: Analysis of HepG2/C3A cell-derived DNA following treatment with ellagic acid. Ten micrograms of *EcoRI*-digested cellular DNA were loaded per lane. DNA blots were probed with [³²P]-labeled HBV cDNA probes. A 1 kb DNA ladder was used to estimate product size. Lane 1 (control), DMSO. Lanes 2–4, 3TC (0.1, 1.0, and 10 mM). Lanes 5–8, ellagic acid (10, 20, 40, and 60 µg/mL)

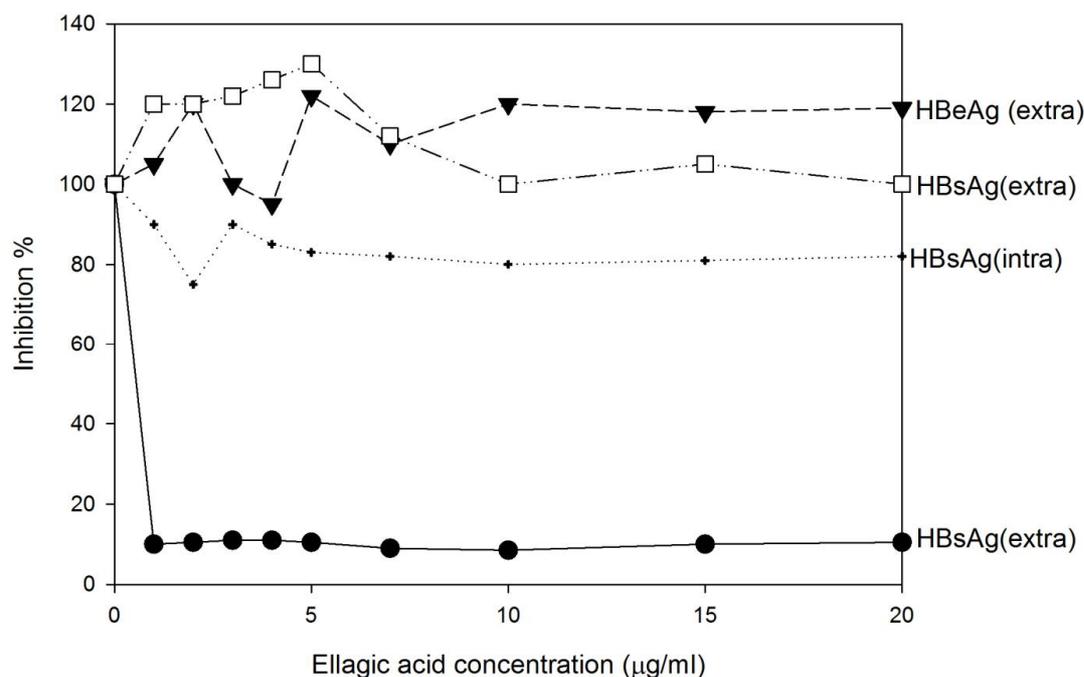


Figure 6: Effect of ellagic acid on HBeAg and HBsAg secretion by HepG2/C3A cells. Ellagic acid inhibited HepG2/C3A HBeAg secretion. HBsAg and HBeAg levels in lysates from HepG2/C3A cells treated with ellagic acid (1–5, 7, 10, 15, and 20 µg/mL)

In this study, *P. niruri* extract exhibited anti-HBV activity in HepG2/C3A cells.

Ellagic acid, found in berry fruits, has components with anti-inflammatory, anti-fibrotic, and antioxidant activities. Ellagic acid was shown to reduce HBeAg production by HepG2 2.2.15 cells [22].

Ellagic acid has potent antioxidant activity. Moreover, ellagic acid has growth-inhibiting and apoptosis-inducing cytotoxic effects on cancer cells, including leukemic, pancreatic, breast, neuroblastoma, colon, prostate, tongue, and osteogenic sarcoma cells. Ellagic acid-treated cells show apoptosis, increased caspase 3/7 activities, activation of caspase 3, and cleavage of poly-ADP ribose polymerase [23]. Although ellagic acid possesses potent activity against human DNA polymerases β , η , ι , and κ *in vitro*, our data did not indicate significant inhibition [24]. This indicates that viral reproduction is not suppressed by ellagic acid. As HBV is a DNA virus, reverse transcriptase inhibitors may be a promising therapeutic to inhibit HBV DNA replication [25].

CONCLUSION

A potential anti-HBV agent, ellagic acid, was isolated from the medicinal plant *P. niruri*, and its physicochemical properties were characterized. The isolated compound exerted a cytotoxic effect

against HepG2/C3A cells. Ellagic acid did not affect HBV replication.

DECLARATIONS

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Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

The authors declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by them.

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