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Piperine, an active ingredient of black pepper attenuates acetaminophen-induced hepatotoxicity in mice

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ABSTRACT

Objective: To explore the hepatoprotective and antioxidant effects of piperine against acetaminophen-induced hepatotoxicity in mice. **Methods:** In mice, hepatotoxicity was induced by a single dose of acetaminophen (900 mg/kg b.w. i.p.). Piperine (25 mg/kg b.w. i.p.) and standard drug silymarin (25 mg/kg b.w. i.p.) were given to mice, 30 min after the single injection of acetaminophen. After 4 h, the mice were decapitated. Activities of liver marker enzymes [(aspartate transaminase (AST), alanine transaminase (ALT), and alkaline phosphatase (ALP)] and inflammatory mediator tumour necrosis factor- α (TNF- α) were estimated in serum, while lipid peroxidation and antioxidant status (superoxide dismutase, catalase, glutathione peroxidase, glutathione reductase, glutathione-s-transferase and glutathione) were determined in liver homogenate of control and experimental mice. **Results:** Acetaminophen induction (900 mg/kg b.w. i.p.) significantly increased the levels of liver marker enzymes, TNF- α , and lipid peroxidation, and caused the depletion of antioxidant status. Piperine and silymarin treatment to acetaminophen challenged mice resulted in decreased liver marker enzyme activity, TNF- α and lipid peroxidation levels with increase in antioxidant status. **Conclusions:** The results clearly demonstrate that piperine shows promising hepatoprotective effect as comparable to standard drug silymarin.

1. Introduction

Acetaminophen is an over the counter (OTC) drug commonly used for its analgesic and antipyretic properties. Even though it is considered a safe drug, overdose or by chronic use, it cause hepatic damage, particularly in the presence of predisposing factors such as chronic alcohol consumption[1]. During therapeutic doses, acetaminophen is metabolized normally by the cytochrome P450 system, which leads to the formation of n-acetyl-p-benzoquinoneimine (NAPQI). However, overdose of acetaminophen causes depletion of the cellular glutathione level in liver; because NAPQI reacts rapidly react with glutathione, which leads to oxidative stress, cell damage and death[2]. Since liver disease is a widespread pathology and currently used hepatoprotective drugs are of doubtful efficacy and safety. It is therefore, necessary to search for alternative drugs for the treatment of liver disease. In spite of significant advances in medicinal plant research, there has been a shift towards therapeutic evaluation of herbal products in liver diseases. Thus, the development of new hepatoprotective drug of

greater effectiveness and safety is highly warranted.

Piperine (Figure 1) is an alkaloid present as the major pungent ingredient in various parts of the plants from the family *Piperaceae*[3]. Piperine is primarily found in the fruit of the pepper vine, piper nigrum. The pepper vine is indigenous to the Malabar Coast of India, but is also grown in southern Asia, South America and Africa. Piperine exhibits a wide variety of biological effects including anti-metastatic[4], antithyroid[5], anti-depressant[6], hepatoprotective against CCl₄-induced toxicity[7], anti-tumour and immunostimulating activity[8]. Piperine is reported to have anti-anxiety and anti-inflammatory activity[9]. In addition, piperine also act as bioavailability enhancer for various structurally and therapeutic diverse drugs[10]. However, scientific literature supporting the use of the piperine in acetaminophen-induced hepatotoxicity is not available. Therefore, the present study was aimed to evaluate the potential of piperine against acetaminophen-induced hepatotoxicity in mice.

2. Materials and methods

2.1. Animals

Male cross breed Swiss albino mice weighing about 20–25 g

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were purchased from the Karigiri Hospital and Research Centre, Vellore, India. The animals were housed in large spacious cages. They were acclimatized for a week in a light and temperature-controlled room with a 12 h dark-light cycle and fed with commercial pelleted feed Hindustan Lever Ltd. (Mumbai, India) and water *ad libitum*. The animals used in this study were treated and cared for well being in accordance with the guidelines recommended by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Culture, Government of India, Chennai, India. The experimental protocol was also approved by our departmental ethics committee.

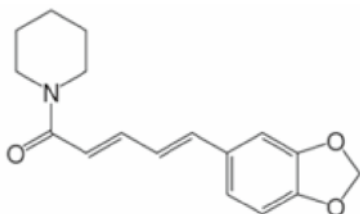


Figure 1. Chemical structure of piperine (1-[5-(1,3-Benzodioxol-5-yl)-1-oxo-2,4-pentadienyl] piperidine ($C_{17}H_{19}NO_3$).

2.2. Drugs and chemicals

The commercially available piperine (Light yellow powder, > 98% purity by HPLC) was obtained from the Natural Remedies Pvt. Ltd, Bangalore, India. Silymarin, a standard hepatoprotective drug and acetaminophen were obtained from the Micro Labs Ltd, Goa, India. All other reagents and chemicals used were of analytical grade.

2.3. Dose determination study

A preliminary study was carried out to determine the optimum dose of piperine by investigating serum hepatic marker enzyme activities in acetaminophen-induced rats. Piperine suspended in a saline was administered at different doses (*i.e.* 15, 20 and 25 mg/kg b.w.) to different groups of animals. Among the three doses, the 25 mg/kg b.w. was more effective than the other two doses. Hence 25 mg/kg b.w. dose was considered for this study. The dosage of acetaminophen (900 mg/kg b.w.) and standard drug silymarin (25 mg/kg b.w.) used in this study were selected based on our previous reports^[11].

2.4. Experimental protocol

In this experiment, mice were randomly allocated into 5 groups, each consisting of six animals. All animals were kept fasting for 24 h before the experiment. The 1st group, the control group, received saline. The 2nd group, acetaminophen group was treated with a single dose of acetaminophen (900 mg/kg b.w. i.p.)^[12]. Acetaminophen was first dissolved in water at 70 °C, and then cooled to 37 °C before administration. The 3rd group, (piperine +paracetamol) was given piperine (25 mg/kg b.w. i.p. dissolved in saline) with a single dose of acetaminophen (900 mg/kg b.w. i.p.). The 4th group (Silymarin+acetaminophen) was given silymarin (25 mg/kg b.w. i.p. dissolved in saline) 30 min after the single injection of acetaminophen. The 5th group, piperine group, received piperine alone (25 mg/kg b.w. i.p. dissolved in saline). The

dose selection for acetaminophen, silymarin, and piperine was based on our preliminary and previous experiments^[11]. The mice were decapitated 4 h after the acetaminophen injection; the trunk blood was collected, the serum was separated and stored at -70 °C. Tissue samples from the liver were obtained for biochemical analysis.

2.5. Biochemical parameters

The activities of alkaline phosphatase (ALP), alanine and aspartate aminotransferases (AST, ALT) in serum were estimated by using commercial kits (Span Diagnostics, Chennai, India).

In the hepatic tissue samples, Lipid peroxidation was measured using thiobarbituric acid reactive substances (TBARS) according to the method of Ohkawa *et al*^[13]. The lipid peroxidation was expressed as μ moles of TBA reactants/100 g of tissue homogenate. Superoxide dismutase (SOD) was estimated in liver homogenate according to the method of Marklund *et al*^[14]. Catalase (CAT) was assayed by the method of Sinha^[15]. Glutathione peroxidase (GPx) was assayed by the method of Rotruck *et al*^[16]. Glutathione reductase (GR) that utilizes NADPH to convert oxidized glutathione (GSSG) to the reduced form was assayed by the method of Bellomo *et al*^[17]. Glutathione-S-transferase (GST) was assayed by the method of Habig *et al*^[18]. Total reduced glutathione (GSH) was determined by the method of Moron *et al*^[19]. The protein content was determined by the method of Lowry *et al*^[20] using bovine serum albumin as a standard. TNF- α level in serum of control and experimental mice were determined by enzyme-linked immunosorbent assay (ELISA, Cayman Chemicals, USA), according to the manufacturer's instructions.

2.6. Histopathological studies

Immediately after sacrifice, a portion of the liver was fixed in 10% formalin, then washed dehydrated in descending grades of isopropanol and finally with xylene. The tissue was then embedded in molten paraffin wax. Sections were cut at 5 μ m thickness, stained with haematoxylin and eosin was observed microscopically for histopathological changes.

2.7. Statistical analysis

Results were expressed as mean \pm SD and statistical analysis was performed using ANOVA, to determine the significant differences between the groups, followed by Student's Newman-Keul's test, $P < 0.05$ implied significance.

3. Results

Table 1 shows the effect of piperine on TBARS formation, superoxide dismutase, catalase, glutathione peroxidase, glutathione reductase, glutathione-S-transferase and glutathione in liver of control and experimental mice. In acetaminophen treated mice, TBARS level was increased significantly; whereas superoxide dismutase, catalase, glutathione peroxidase, glutathione reductase, glutathione-S-transferase and glutathione were found to be decreased when compared to the control group. However, piperine treatment to acetaminophen induced mice altered the above changes by regulating the TBARS level and anti-oxidant

enzymes to nearly that of normal levels.

Figure 2 & 3 shows the activities of alanine and aspartate transaminases, and alkaline phosphatase in serum of normal, acetaminophen control and treated groups. The activities of alanine transaminase, aspartate transaminase, and alkaline phosphatase in serum were significantly increased in acetaminophen control group compared to normal control group. However, the administration of piperine to acetaminophen-induced mice prevented the above changes observed in acetaminophen-induced mice.

Figure 4 shows the levels of pro-inflammatory cytokine tumour necrosis factor- α (TNF- α) in the serum of control and experimental animals. A significant elevation in the level of TNF- α were noticed in the serum of acetaminophen treated mice, however the elevated level of TNF- α was found to be reduced in piperine administered mice treated with acetaminophen.

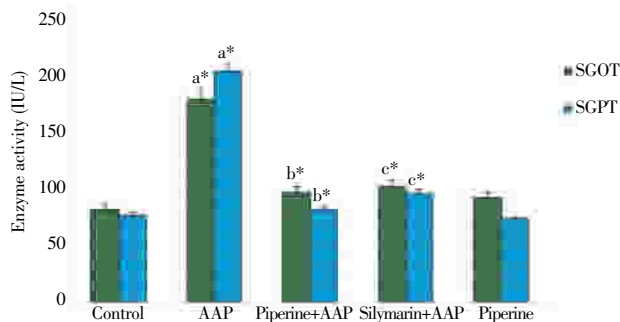


Figure 2. Hepatoprotective effect of piperine on liver functional markers (SGPT & SGOT) levels in AAP-intoxicated mice. SGPT & SGOT levels in serum samples of the control, acetaminophen (AAP), Piperine+AAP, Silymarin+AAP and Piperine groups. Each value is expressed as mean \pm SD of six animals. Comparisons were made as follows: a-control vs AAP; b-AAP vs piperine+AAP; c-AAP vs silymarin+AAP. The symbols represent statistical significance at: * P <0.05. Statistical analysis was calculated by one way ANOVA followed by Student's Newman-Keul's test.

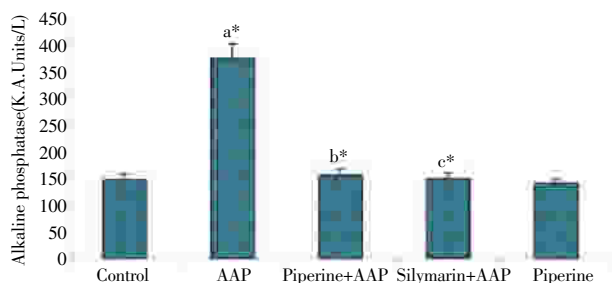


Figure 3. Hepatoprotective effect of piperine on serum alkaline phosphatase levels in AAP-intoxicated mice. Alkaline phosphatase levels in serum samples of the control, AAP, Piperine+AAP, Silymarin+AAP and Piperine groups. Each value is expressed as mean \pm SD of six animals. Comparisons were made as follows: a-control vs AAP; b-AAP vs piperine+AAP. c-AAP vs silymarin+AAP. The symbols represent statistical significance at: * P <0.05. Statistical analysis was calculated by one way ANOVA followed by Student's Newman-Keul's test.

Figure 5 presents the histology of liver of control and experimental mice. Light microscopic evaluation showed regular morphology of liver with well designed hepatic cells in control and piperine (25 mg/kg b.w. i.p.) alone treated

groups (Figure 5a & 5e). Acetaminophen (900 mg/kg b.w. i.p.) treated group showed extensive microvesicular degenerative change and necrosis in some hepatocytes (Figure 5b). The histopathological pattern of the liver of the acetaminophen-induced mice treated with piperine (25 mg/kg b.w. i.p.) and silymarin (25 mg/kg b.w. i.p.) showed no necrosis and some hepatocytes with condensed nuclei (Figure 5c & 5d).

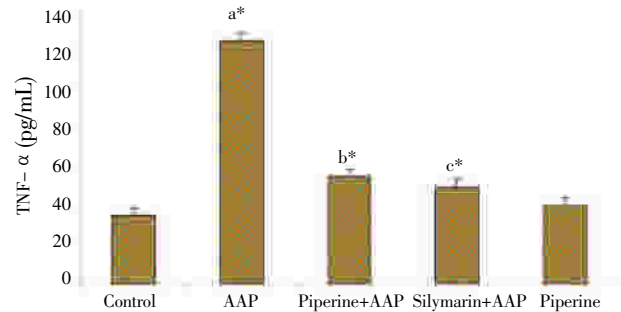


Figure 4. Hepatoprotective effect of piperine on serum TNF- α levels in acetaminophen intoxicated mice. TNF- α level in serum samples of the control, AAP, Piperine+AAP, Silymarin+AAP and Piperine groups. Each value is expressed as mean \pm SD of six animals. Comparisons were made as follows: a-control vs AAP; b-AAP vs piperine+AAP; c-AAP vs silymarin+AAP. The symbols represent statistical significance at: * P <0.05. Statistical analysis was calculated by one way ANOVA followed by Student's Newman Keul's test.

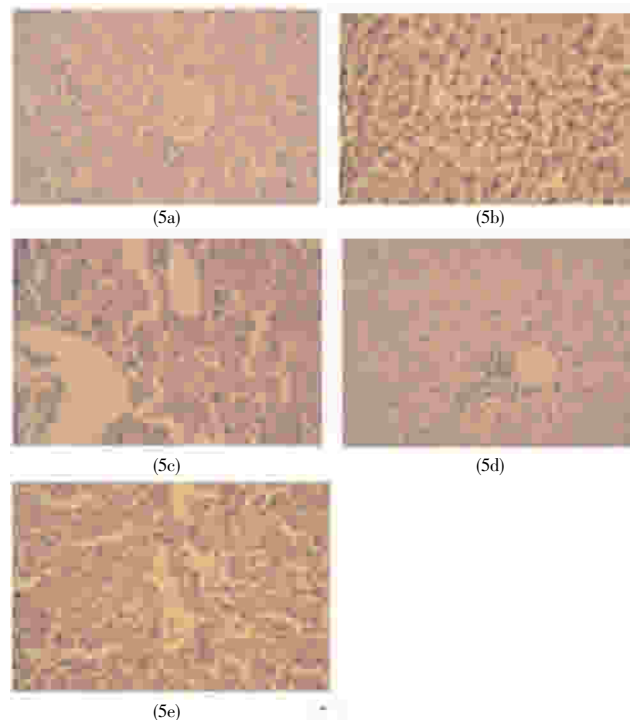


Figure 5. Hepatoprotective effect of piperine and silymarin against acetaminophen-induced mice. Photomicrographs of liver sections taken from different groups. (5a) control group shows normal lobular architecture and normal hepatocytes, (5b) AAP (900 mg/kg b.w. i.p.) group shows degenerative change in cytoplasm and necrosis in hepatic cell, (5c) AAP+piperine (25 mg/kg b.w. i.p.) shows marked improvement over AAP treated group, (5d) AAP group+silymarin (25 mg/kg b.w. i.p.) shows almost normal architecture of liver and (5e) Piperine control (25 mg/kg b.w. i.p.) shows normal lobular architecture and normal hepatocytes (Haematoxylin & Eosin staining, original magnification 400 \times).

Table 1

Effect of piperine (25 mg/kg b.w.) and silymarin (25 mg/kg b.w.) on liver lipid peroxidation levels and antioxidant enzyme activities in acetaminophen-intoxicated mice.

Parameters	Control	Acetaminophen (900 mg/kg b.w. i.p.)	Acetaminophen+piperine (25 mg/kg b.w. i.p.)	Acetaminophen+silymarin (25 mg/kg b.w. i.p.)	Piperine (25 mg/kg b.w. i.p.)
Lipid peroxidation– (μ mole of TBA reactants/100 g tissue)	1.26±0.09	2.63±0.21a*	1.55±0.13b*	1.68±0.16c*	1.20±0.18
Superoxide dismutase – (Units/mg protein/min)	234.67±15.97	116.83±7.72a*	217.50±2.43b*	212.17±17.68c*	224.14±16.30
Catalase –(μ mol of H ₂ O ₂ consumed/min/mg protein)	12.68±1.22	7.16±0.42a*	10.83±0.77b*	9.86±0.82c*	11.00±0.96
Glutathione peroxidase – (μ g of GSH utilized/min/ mg protein)	75.08±8.34	34.75±2.68a*	65.35±5.02b*	63.51±5.67c*	73.58±8.07
Glutathione reductase – (nmol of NADPH oxidized/ min/mg protein)	122.43±10.99	66.83±5.56a*	107.70±8.28b*	100.43±8.10c*	117.27±9.72
Glutathione–S– transferase–(nmol of 1–chloro–2,4– dinitrobenzene–GSH conjugate formed/min/mg protein)	91.98±6.12	69.16±5.68a*	83.18±5.94b*	80.85±6.70c*	88.35±5.61
Total reduced glutathione (nmol/mg/protein)	45.68±3.26	23.41±1.95a*	41.05±2.93b*	38.88±2.87c*	43.18±2.29

Comparisons were made as follows: a–control vs AAP; b–AAP vs piperine+AAP; c–AAP vs silymarin+AAP. The symbols represent statistical significance at: * P <0.05. Statistical analysis was calculated by one way ANOVA followed by Student's Newman–Keul's test.

4. Discussion

Overdose of acetaminophen, a popular antipyretic, and analgesic, is a leading cause of acute liver damage resulting in approximately 1 800 deaths per year in the United States. Several studies have established liver repair and regeneration as a critical determinant in the final outcome of toxicant-induced liver injury^[21]. In this study, we evaluated the role of piperine against acetaminophen-induced hepatic damage in mice. An obvious sign of hepatic injury is the leaking of cellular enzymes into the plasma due to the disturbance caused by in the transport functions of the hepatocytes^[22]. The estimation of enzymes in the serum is useful quantitative marker of the extent and type of hepatocellular damage. In agreement with previous studies that, acetaminophen intraperitoneal injection increased serum ALT, AST activities and exacerbated oxidative injury^[2]. In the present study, administration of acetaminophen caused a significant elevation of enzyme levels such as AST, ALT, ALP and lipid peroxidation when compared to the control group. The reversal of increased serum enzymes and lipid peroxidation in acetaminophen-induced liver damage by piperine may be due to the prevention of the leakage of intracellular enzymes by its membrane stabilizing and antioxidant activity, which was supported by the limited extent of histological changes. This is in agreement with the commonly accepted view that serum levels of transaminases return to normal with the healing of hepatic parenchyma and the regeneration of hepatocytes^[23]. There is extensive evidence that piperine supplementation can enhance

antioxidant enzymes and other selenoproteins^[24].

It is well established that acetaminophen is bioactivated to N–acetyl–p–benzoquinone, which increase the formation of reactive nitrogen species. Excess level of reactive oxygen species can attack biological molecules such as DNA, protein, and phospholipids, which leads to excess lipid peroxidation and depletion of the glutathione and antioxidant enzymes that further result in oxidative stress^[25]. The NAPQI induced depletion of cytosolic and mitochondrial GSH trigger the loss of cellular homeostasis leading to liver injury^[26]. The activity of SOD is a sensitive index in hepatorenal damage as it scavenges the superoxide anion to form hydrogen peroxide leading to diminish the toxic effects. The main detoxifying systems for peroxides in hepatic cells are catalase and glutathione^[27]. Catalase is an antioxidant enzyme, which destroys H₂O₂ that can form a highly reactive hydroxyl radical in presence of iron as a catalyst^[28]. By participating in the glutathione redox cycle, Glutathione together with glutathione peroxidase convert H₂O₂ and lipid peroxides to non-toxic products. Glutathione reductase and glutathione–s–transferase are thought to be the fundamental antioxidant enzymes, for they are closely related to the direct elimination of reactive oxygen species. Therefore, the reduction in the activity of these enzymes may result in a number of deleterious effects due to the accumulation of superoxide radicals and hydrogen peroxide^[29]. In consistence with our previous report^[11], in this present study, acetaminophen induction (900 mg/kg b.w.) significantly increases the levels of inflammatory mediator TNF– α , lipid peroxidation, and caused the depletion

of antioxidant status (superoxide dismutase, catalase, glutathione peroxidase, glutathione reductase, glutathione-S-transferase and glutathione). However, treatment with piperine abrogated the acetaminophen-induced decrease in antioxidant enzymes and glutathione in mice. This indicates that involvement of piperine in facilitating the rapid and efficient consumption of reactive oxygen species generated by acetaminophen P450 bioactivation. Earlier studies from other laboratories have reported that piperine treatment lowered lipid peroxidation *in vivo* and beneficially influenced cellular thiol status, antioxidant molecules and antioxidant enzymes against chemical induced oxidative stress in various pathological diseases^[30,31]. Moreover piperine itself acts as an antioxidant through its radical quenching effect and prevented the depletion of glutathione level^[32]. Glutathione, a non-enzymic antioxidant is well known against chemically induced toxicity through its participation in the removal of reactive intermediates by conjugation, hydroperoxide reduction or by direct quenching of free radicals^[33]. In our study, piperine administration was shown to elevate the glutathione contents in liver cells of acetaminophen-induced mice. Therefore, our result suggests that possible mechanism of action of piperine against free radicals is through by maintaining high pool of glutathione either by decreasing its catabolism or increasing the transport or synthesis. In addition, with regard to structure-activity relationships, the amide/phenol group present in the structure of piperine might be responsible for its free radical scavenging activity^[34].

TNF- α , a proinflammatory cytokine is important in regulating hepatocyte proliferation, tissue repair, and matrix remodeling in various models of liver injury^[35]. TNF- α mediates a number of organ injury through its induction of cellular apoptosis. In the liver, the biological effects of TNF- α have been implicated in hepatic injury induced hepatic toxins, ischemia/reperfusion, viral hepatitis, and alcohol^[34]. The production of TNF- α is known to be one of the earliest events in the hepatic inflammatory response, which induce cytotoxicity, hepatocyte apoptosis and necrosis^[36]. Therefore, TNF- α is considered as an important target in research to discover hepatoprotective agents. In accordance to this report, our results demonstrate that acetaminophen increases serum TNF- α , indicating the role of this cytokine in acetaminophen-induced hepatotoxicity. However, piperine administration significantly reduced the elevated TNF- α in acetaminophen treated mice.

In conclusion, the results of this study demonstrate that piperine has a potent hepatoprotective effect on acetaminophen-induced hepatic damage in mice due to its radical quenching effect. However, further pharmacological evidences at molecular level are required to establish the actual mechanism of the action of the drug which is underway.

Conflict of interest statement

We declare that we have no conflict of interest.

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Letter to editor

Emerging of dengue infection after big flood in Thailand: a concern

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Editor, dengue is an important tropical borne mosquito infection. This infection is very common in Southeast Asia especially for Thailand and nearby country. There are several factors that lead to the emerging of dengue infection. Here, the author presents the interesting observation on emerging of dengue infection after big flood in Thailand. In mid 2010, big flood occurs around Thailand. About 30% of total area of Thailand was occupied by water. Interestingly, a sharp increase of dengue prevalence was reported in many provinces. Up to 83% increasing of prevalence was noted by Thai Minister of Public Health (<http://thairecent.com/Breaking/2010/695901/>).

It can be shown that the flood as natural disaster is an important crisis that leads to the emerging of dengue infection. This observation is very interesting. There is no systematical study to assess the effect of flooding on dengue prevalence. Indeed, heavy rainfall is proved to be an important factor relating to the emerging of dengue[1]. The change of ambient temperature and humidity concordant with heavy rainfall during flood period is believed to be the determinant of the prevalence of dengue[2].

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