Anti-inflammatory and in vitro Antioxidant Property of Zanthoxylum nitidum Root

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Abstract:

Zanthoxylum nitidum (Roxb.) DC (Rutaceae), called Tez-mui or Tejamool in Assamese is a large prickly shrub occurring in North-East India and its roots are used traditionally for several medicinal purposes. In present study the methanol extract of roots from Zanthoxylum nitidum (ZNME) was evaluated for its anti-inflammatory activity in acute (carrageenan, histamine and serotonin induced rat paw oedema) and chronic models (cotton pellet induced granuloma). In all models the ZNME (75 and 150 mg/kg body wt. p.o.) exhibited significant anti-inflammatory activity (p < 0.001) in a dose dependent manner when compared with saline control. Indomethacin (10 mg/kg body wt. p.o.) was used as reference drug. The ZNME was evaluated for its antioxidant properties by 1,1-diphenyl-2-picryl hydrazyl (DPPH) radical scavenging assay and in vitro lipid peroxidation induced by the Fe²⁺- ascorbate system in rat liver homogenate. In DPPH radical scavenging assay, the ZNME demonstrated marked and dose dependent free radical scavenging effect and the mean inhibitory concentration (IC₅₀) of the ZNME was found to be 75.2 μg/ml, while the ascorbic acid (reference) exhibited 43.7 μg/ml. The ZNME effectively inhibited the lipid peroxidation in a dose related manner showing the IC₅₀ value of 279.1 μg/ml, whereas the quercetin (reference) showed 46.6 μg/ml. These findings revealed that the Z. nitidum root had remarkable acute and chronic anti-inflammatory and in vitro antioxidant actions in the tested models validating its traditional uses.

Key words: Zanthoxylum nitidum, anti-inflammatory, radical scavenging, lipid peroxidation.

Introduction

Zanthoxylum nitidum (Roxb.) DC (Rutaceae), called Tez-mui or Tejamool in Assamese, is a morphologically variable plant species occurring in South-East Asian countries and in Northern Australia (1). In India it grows as a large prickly shrub particularly in North-East India (Sikkim, Assam and Nagaland states). In India the plant has traditionally been used for various medicinal purposes. The root is used in toothache, stomachache, fever, rheumatism, paresis, boils and as an insecticide and piscicide. The fruit is used in the treatment of stomachache, cough, colic, vomiting, diarrhoea, and paresis and as an aromatic, stimulant and piscicide. The small branches, seeds and stem bark are prescribed in fever, diarrhoea and cholera (2-4). It has come to the author’s notice that the rural people of upper Assam, India use the young stems of this plant as chewing stick in treatment of toothache and gingivitis. Previously the authors have reported

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essential oil composition of fruits and leaves, pharmacognostic parameters of stem bark and root, antibacterial effects of stem bark and root, anti-nociceptive activity of stem bark of Z. nitidum from India (5-9). There are no reports of anti-inflammatory investigations carried out on Z. nitidum of Indian habitat. The present work therefore, attempts to report the preliminary results of studies on anti-inflammatory and in vitro antioxidant effects of Z. nitidum root in experimental models to justify the traditional and folkloric beliefs.

Materials and Methods

Plant material: The mature entire plants of Z. nitidum were collected during the month of November 2007 from Dibrugarh district of Assam state, India. The species was identified by Dr. S. J. Phukan, taxonomist, from Botanical Survey of India, Eastern Circle, Shillong, India, and a voucher specimen (No. DUPS-06-003) was deposited in Department of Pharmaceutical Sciences, Dibrugarh University for future reference. The roots were separated from the aerial parts and cut into small pieces. Then the plant material was shade dried at temperature 21-24 °C and ground mechanically into a coarse powder and stored in an air-tight container.

Preparation of extract: Powdered plant material (150 g) was macerated with 400 ml of methanol at 21-24 °C temperature for 2 days with frequent shaking. After 2 days, the extracts were filtered and to the marc part 300 ml of the solvent was added and allowed to stand for next 2 days at same temperature for second time maceration (re-maceration) and after two days, again filtered similarly. The combined filtrates (macerates) were evaporated in vacuo at 40 °C and the dry extract obtained (ZNME, yield 11.6 % w/w) was stored in a vacuum desiccator for future use. Preliminary phytochemical studies indicated the presence of alkaloids, flavonoids, carbohydrates, reducing sugars and amino acids in ZNME (10).

Drugs and chemicals: λ-Carrageenan (type IV) was obtained from S. D. Fine Chemicals Ltd., Bombay; 5-hydroxytryptamine hydrochloride (serotonin), histamine sulphate, thiobarbituric acid were from Sigma Chemical Co., USA; indomethacin was from Recon, Bangalore, India; DPPH, L-ascorbic acid were from Sisco Research Laboratories Pvt. Ltd., India; quercetin was from Aldrich Chemical Co., UK. All other chemicals, reagents and solvents were of analytical grade available commercially.

Animals: Studies were carried out using adult male Wistar albino rats of weighing 150-180 g. They were obtained from the animal supplier (Ghosh & Co., Kolkata, India). The animals were grouped in polyacrylic cages (38 cm × 23 cm × 10 cm) with not more than four animals per cage and maintained under standard laboratory conditions (temperature 25 ± 2 °C, dark and light cycle 14/10 h). They were allowed free access to standard dry pellet diet (Hindustan Lever, Kolkata, India) and water ad libitum. The rats were acclimatized to laboratory condition for 10 days before commencement of experiment. All experimental methods were approved by University Animal Ethical Committee, Jadavpur University (Reg. no. 367001/C/CPCSEA).

Acute toxicity: The oral LD₅₀ value of ZNME in male Swiss albino mice were determined as per reported method (11).

Anti-inflammatory activity

Carrageenan-induced rat paw oedema: The rats were divided into four groups (n = 6). The first group (which served as control) received normal saline (0.9 % w/v, 3 ml/kg body wt., p.o.). The second and third group received the test
extract ZNME (75 and 150 mg/kg body wt., p.o., respectively). The fourth group (which served as reference) received indomethacin (10 mg/kg body wt., p.o.). After 30 mins, acute inflammation was produced by the subplantar administration of 0.1 ml of 1% (w/v) of freshly prepared suspension of carrageenan in the right hind paw of each rat. The paw volume was measured at 0 h and 3 h after carrageenan injection by using plethysmometer (Ugo Basile, Italy). The difference between the two readings was taken as the volume of oedema and the percentage of inhibition was calculated (12-14).

Mediator-induced inflammation: The paw oedema was induced in rats by subplantar injection of 0.1 ml of freshly prepared histamine (1 mg/ml) and serotonin (1 mg/ml) solutions respectively (15,16). Group division and treatment regime of the animals were same as the carrageenan induced rat paw oedema model and the paw oedema was measured as mentioned earlier.

Cotton pellet-induced granuloma: The animals were divided into four groups (n = 6). The rats were anaesthetized and sterile cotton pellets weighing 10 ± 1 mg were implanted subcutaneously into both sides of the groin region of each rat. The first group (which served as control) received normal saline (0.9% w/v, 3 ml/kg body wt., p.o.). The second and third group received the test extract ZNME (75 and 150 mg/kg body wt., p.o., respectively). The fourth group (which served as reference) received indomethacin (10 mg/kg body wt., p.o.). All groups were treated in this way for seven consecutive days from the day of cotton pellet implantation (17). On 8th day the animals were anaesthetized and the pellets together with the granuloma tissues were carefully removed and made free from extraneous tissues. The wet pellets were then dried in an oven at 60 °C for 24 h to constant weight. Increment in the dry weight of the pellets was taken as a measure of granuloma formation (18).

In vitro antioxidant property
Scavenging activity of DPPH: The antioxidant property of ZNME was determined on the basis of their scavenging activity of stable 1,1-diphenyl-2-picryl hydrazyl (DPPH) free radical (19, 20). Briefly, 0.1 mM solution of DPPH in methanol was prepared and 1 ml of this solution was added to 3 ml of ZNME suspension in water at different concentrations (25-200 μg/ml). The mixture was shaken and allowed to stand at room temperature for 30 min and the absorbance was measured at 517 nm using methanol as blank on UV-visible spectrophotometer Shimadzu, UV-1601. The scavenging activity was measured as the decrease in absorbance of the samples versus standard DPPH solution. Ascorbic acid was used as the reference. The results were expressed as percentage of inhibition at different concentrations and IC_{50} was determined. The IC_{50} (mean inhibitory concentration) value denotes the concentration of the sample (in μg/ml) required to scavenge 50% of the DPPH free radicals. The percentage scavenging activity was calculated by using the following formula,

\[
\left( \frac{A_{\text{control}} - A_{\text{sample}}}{A_{\text{control}}} \right) \times 100,
\]

Where \( A_{\text{control}} \) is the absorbance of the control reaction (containing all reagents except the test extract) and \( A_{\text{sample}} \) is the absorbance of the sample at different concentrations. All the tests were performed in triplicate and the results averaged.

Determination of inhibition of lipid peroxidation
Tissue sample preparation: The liver of normal rat was excised and perfused in vitro with ice cold normal saline (0.9% w/v). The tissues were then homogenized at a concentration of 10% w/

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v in 1.15 % w/v KCl solution and centrifuged at 1200 g at 4 ºC for 10 mins. The supernatant was collected which was again centrifuged at 10000 g at - 4ºC for 10 mins. The supernatant was taken and stored at -20ºC for use in the study (21).

Estimation method: Lipid peroxidation induced by Fe²⁺- ascorbate system in rat liver homogenate was estimated as thiobarbituric acid reacting substance (TBARS) by the method of Ohkawa et al (22). The reaction mixture contained rat liver homogenate 0.1 ml in Tris-HCl buffer (40 mM, pH 7.0); KCl (30 mM); FeSO₄(NH₄)₂SO₄_7H₂O (0.16 mM); sodium ascorbate (0.06 mM); and various concentrations of ZNME in a final volume of 0.5 ml. The reaction mixture was incubated at 37ºC for 1 h. After the incubation period, 0.4 ml was removed and treated with 0.2 ml sodium dodecyl sulphate (SDS, 8.1%); 1.5 ml thiobarbituric acid (TBA, 0.8%); and 1.5 ml acetic acid (20%, pH 3.5). The total volume was made up to 4 ml with distilled water and then kept in a water bath at 95 to 100°C for 1 h. After cooling, 1.0 ml of distilled water and 5.0 ml of n-butanol and pyridine mixture (15: 1 v/v) were added to the reaction mixture, shaken vigorously and centrifuged at 4000 rpm for 10 min. The upper n-butanol-pyridine layer was removed and its absorbance at 532 nm was measured by using UV-visible spectrophotometer Shimadzu, UV-1601. Inhibition of lipid peroxidation was determined by comparing the absorbance of treatments with that of the control. Quercetin was used as the reference.

The inhibitory ratio of the test sample was evaluated by the following formula,

\[
\text{Percentage inhibition} = \frac{A_c - A_s}{A_c} \times 100 \%
\]

Where \(A_c\) is the absorbance of control (containing all reagents except the test extract) and \(A_s\) is the absorbance of the sample at different concentrations. All the tests were performed in triplicate and the results averaged.

Statistical analysis: Except antioxidant studies the values were expressed as mean ± standard error of mean (SEM). The statistical significance was determined by using the Student’s ‘t’ test (23). Values of \(p < 0.001\) were considered as statistically significant.

Results

The oral LD₅₀ value of ZNME in mice was found to be 1500 mg/kg body weight.

The anti-inflammatory activity of Z. ntidum root extract (ZNME) was evaluated against carrageenan induced acute paw oedema in rats and the results are summarized in Table 1. The ZNME produced significant (\(p < 0.001\)) anti-inflammatory activity in a dose dependent manner.

| Table 1. Effect of ZNME on carrageenan induced rat paw oedema |
|-----------------|--------------|----------------|----------------|
| Treatment       | Dose (mg/kg) | Increase in paw volume (ml) ± SEM | Percentage of inhibition |
| Control         | -            | 0.774 ± 0.13 | -              |
| ZNME            | 75           | 0.483 ± 0.08*| 43.67          |
| ZNME            | 150          | 0.332 ± 0.04*| 57.11          |
| Indomethacin    | 10           | 0.219 ± 0.02*| 71.71          |

Number of animals per group (\(n\)) = 6. SEM = Standard Error of Mean. *\(p < 0.001\), compared to control group.
The ZNME showed maximum inhibition of 57.11% at the dose of 150 mg/kg body wt. after 3 h of treatment, whereas the reference drug indomethacin produced 71.71% of inhibition.

In case of histamine and serotonin induced paw oedema, ZNME exhibited significant ($p < 0.001$) and dose dependent protection from oedema (Table 2 and 3). The ZNME produced 51.89% inhibition in case of histamine and 59.66% of inhibition in case of serotonin at the dose of 150 mg/kg body wt.; while the reference drug, indomethacin produced 62.37 and 70.63% of inhibition of paw oedema respectively in above two mediators.

In chronic inflammatory model (cotton pellet induced granuloma) the ZNME significantly ($p < 0.001$) and dose dependently reduced the weight of cotton pellets as compared to the vehicle control (Table 4). The ZNME produced the maximum inhibition of 53.99% at the dose of 150 mg/kg body wt. and the reference drug indomethacin produced 64.06% of inhibition of granuloma formation.

The antioxidant property of ZNME was evaluated by DPPH radical scavenging assay and the results are summarized in Table 5. The percentage of inhibition was found to be 64.23% at the concentration of 200 μg/ml and 9.18% at the concentration of 25 μg/ml. The IC$_{50}$ value of ZNME was 75.2 μg/ml. Ascorbic acid was used as reference and its IC$_{50}$ value was found to be 43.7 μg/ml.

The antioxidant property of ZNME was also evaluated by the inhibition of

### Table 2. Effect of ZNME on histamine induced rat paw oedema.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dose (mg/kg)</th>
<th>Increase in paw volume (ml) ± SEM</th>
<th>Percentage of inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>-</td>
<td>0.582 ± 0.024</td>
<td>-</td>
</tr>
<tr>
<td>ZNME</td>
<td>75</td>
<td>0.341 ± 0.008*</td>
<td>41.41</td>
</tr>
<tr>
<td>ZNME</td>
<td>150</td>
<td>0.280 ± 0.015*</td>
<td>51.89</td>
</tr>
<tr>
<td>Indomethacin</td>
<td>10</td>
<td>0.219 ± 0.006*</td>
<td>62.37</td>
</tr>
</tbody>
</table>

Number of animals per group ($n$) = 6. SEM = Standard Error of Mean. *$p < 0.001$, compared to control group.

### Table 3. Effect of ZNME on serotonin induced rat paw oedema.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dose (mg/kg)</th>
<th>Increase in paw volume (ml) ± SEM</th>
<th>Percentage of inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>-</td>
<td>0.647 ± 0.007</td>
<td>-</td>
</tr>
<tr>
<td>ZNME</td>
<td>75</td>
<td>0.389 ± 0.011*</td>
<td>39.88</td>
</tr>
<tr>
<td>ZNME</td>
<td>150</td>
<td>0.261 ± 0.005*</td>
<td>59.66</td>
</tr>
<tr>
<td>Indomethacin</td>
<td>10</td>
<td>0.190 ± 0.003*</td>
<td>70.63</td>
</tr>
</tbody>
</table>

Number of animals per group ($n$) = 6. SEM = Standard Error of Mean. *$p < 0.001$, compared to control group.
malondialdehyde formation generated by Fe\textsuperscript{2+}-ascorbate in rat liver homogenate and the results are summarized in Table 6. The percentage of inhibition was 66.98 % and 28.67 % at the concentrations of 1000 \( \mu \)g/ml and 10 \( \mu \)g/ml respectively. The IC\textsubscript{50} value of ZNME was 279.1 \( \mu \)g/ml and that of quercetin (reference) was found to be 46.64 \( \mu \)g/ml.

### Discussion

The present study establishes the significant anti-inflammatory activity of the methanol extract of the root of \textit{Z. nitidum} (ZNME) in both acute and chronic models. Carrageenan-induced oedema has been commonly used as an experimental animal model for acute inflammation and it is believed to be a biphasic response. The early phase (1 – 2 h) of the carrageenan model is mainly mediated by histamine and serotonin (5-HT). The late phase is mediated by bradykinin, leukotrienes, polymorphonuclear cells and prostaglandins produced by tissue macrophages (24). The ZNME produced dose dependent and significant (\( p < 0.001 \)) inhibition of carrageenan-induced paw oedema after a period of 3 h.

The ZNME also significantly (\( p < 0.001 \)) suppressed the inflammation produced by the mediators viz. histamine and serotonin. It indicates that the ZNME inhibits the inflammation caused by carrageenan and mediators.

### Table 4. Effect of ZNME on cotton pouch induced granuloma in rats.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dose (mg/kg)</th>
<th>Increased wt of cotton pellet (mg) ± SEM</th>
<th>Percentage of inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>-</td>
<td>39.23 ± 0.18</td>
<td>-</td>
</tr>
<tr>
<td>ZNME 75</td>
<td></td>
<td>25.72 ± 0.35*</td>
<td>34.44</td>
</tr>
<tr>
<td>ZNME 150</td>
<td></td>
<td>18.05 ± 0.27*</td>
<td>53.99</td>
</tr>
<tr>
<td>Indomethacin 10</td>
<td></td>
<td>14.10 ± 0.38*</td>
<td>64.06</td>
</tr>
</tbody>
</table>

Number of animals per group (\( n = 6 \)). SEM = Standard Error of Mean.

\*\( p < 0.001 \), compared to control group.

### Table 5. Antioxidant property of ZNME on DPPH radical scavenging activity.

<table>
<thead>
<tr>
<th>Test sample</th>
<th>Percentage of inhibition*</th>
<th>IC\textsubscript{50} (( \mu )g/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>ZNME + DPPH</td>
<td>9.18</td>
<td>75.2</td>
</tr>
<tr>
<td>25 ( \mu )g/ml</td>
<td></td>
<td></td>
</tr>
<tr>
<td>50 ( \mu )g/ml</td>
<td></td>
<td></td>
</tr>
<tr>
<td>100 ( \mu )g/ml</td>
<td></td>
<td></td>
</tr>
<tr>
<td>200 ( \mu )g/ml</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ascorbic acid 50 ( \mu )g/ml</td>
<td>51.33</td>
<td>43.7</td>
</tr>
<tr>
<td>100 ( \mu )g/ml</td>
<td>67.02</td>
<td></td>
</tr>
</tbody>
</table>

\* Values are means (\( n = 3 \)).

### Table 6. Antioxidant property of ZNME on Fe\textsuperscript{2+}-ascorbate induced lipid peroxidation.

<table>
<thead>
<tr>
<th>Test sample</th>
<th>Percentage of inhibition*</th>
<th>IC\textsubscript{50} (( \mu )g/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>ZNME 10 ( \mu )g/ml</td>
<td></td>
<td>28.67</td>
</tr>
<tr>
<td>100 ( \mu )g/ml</td>
<td></td>
<td>37.08</td>
</tr>
<tr>
<td>1000 ( \mu )g/ml</td>
<td></td>
<td>66.98</td>
</tr>
<tr>
<td>Quercetin</td>
<td>44.17</td>
<td>279.1</td>
</tr>
<tr>
<td>10 ( \mu )g/ml</td>
<td></td>
<td></td>
</tr>
<tr>
<td>100 ( \mu )g/ml</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\* Values are means (\( n = 3 \)).
The cotton pellet method is widely used to evaluate the exudative and proliferative components of the chronic inflammation (13). Chronic inflammation is a reaction arising when the acute response is insufficient to eliminate the pro-inflammatory agents. Chronic inflammation includes a proliferation of fibroblasts and the infiltration of neutrophils and exudation. Chronic inflammation occurs by the development of proliferative cells. These cells can either spread or remain in granuloma form. The dry weight of the cotton pellets correlates with the amount of the granulomatous tissue formed (25, 26). The ZNME showed significant ($p < 0.001$) and dose dependent anti-inflammatory action in cotton pellet induced granuloma and hence found to be effective in chronic inflammatory conditions. Based on the results it can be concluded that the ZNME possesses anti-inflammatory potential in both acute and chronic phases of inflammation.

The results of the present study indicate that the ZNME has effective degrees of *in vitro* antioxidant activity by the methods employed. It is now well established that free radicals (e.g. superoxide, hydroxyl radical, nitric oxide) and other reactive species (e.g. hydrogen peroxide, singlet oxygen, peroxy nitrite, hypochlorous acid) contribute to the pathology of many disorders including arthritis and connective tissue disorders, ageing, neurodegeneration, chronic inflammation and cancer (27). Free radicals may also be a contributory factor in the function of the immune system (28). Recent studies suggest that the inflammatory tissue damage is due to the liberation of reactive oxygen species (which act as pro-inflammatory agents) from phagocytes invading the inflammation sites (29, 30).

The DPPH test provides information on the reactivity of test extract with a stable free radical. DPPH is stable nitrogen centered free radical containing an odd electron in its structure that can accept an electron or hydrogen radical to become a stable diamagnetic molecule and usually utilized for detection of radical scavenging activity (19). Because of its odd electron DPPH gives a strong absorption at 517 nm in the visible region (deep violet colour). As the electron becomes paired off in presence of a free radical scavenger, the absorption diminishes, thus the resulting decrease in absorbance is stoichiometric with respect to the number of electrons taken up (20). The ZNME exhibited marked and dose dependent free radical scavenging effect in DPPH radical scavenging assay showing the IC$_{50}$ value 75.2 μg/ml.

Lipid peroxidation is a complex process whereby polyunsaturated fatty acids of cellular membranes undergo reaction with reactive oxygen species to yield lipid hydro-peroxides. Ferrous iron can initiate lipid peroxidation by the Fenton reaction as well as accelerating peroxidation by decomposing lipid hydroperoxides into peroxy and alkoxyl radicals which eventually yield numerous carbonyl products such as malondialdehyde (MDA). This lipid peroxidation can be prevented either by reducing the formation of free radicals or by supplying the competitive substrate for unsaturated lipids in the membrane or by accelerating the repair mechanisms of damaged cell membrane. Several natural and synthetic antioxidants are used to prevent the lipid peroxidation (31, 32).

The antioxidant activity of the ZNME was further confirmed by evaluating the inhibition in production of malondialdehyde (MDA) and related carbonyl products that are produced as by products of lipid peroxidation induced by Fe$^{2+}$-ascorbate system in the biomembranes of rat liver homogenate. These carbonyl products are responsible for DNA damage, carcinogenesis and aging related diseases (33). The MDA reacts with thiobarbituric acid in specific reaction medium to give...
produce a strong absorption at 532 nm. The ZNME effectively inhibited the lipid peroxidation in a dose related manner exhibiting the IC_{50} value of 279.1 μg/ml. This activity is perhaps related to the H+ ion donating capability of the extract, which can scavenge the peroxyl radical to inhibition or termination of the peroxidation chain (22).

Preliminary phytochemical analysis indicated the abundance of true alkaloids and flavonoids in ZNME. Its anti-inflammatory and antioxidant potential could be attributable to these putative constituents. Flavonoids are well known natural antioxidants due to their electron donating property which either scavenge the principal propagating free radicals or halt the radical chain (34). Thus the antioxidant activity of ZNME may be due to the presence of flavonoids which augmented the anti-inflammatory action.

**Conclusion**

Present investigation confirms significant acute and chronic anti-inflammatory and *in vitro* antioxidant properties of the ZNME in the tested models. It can be inferred that the anti-inflammatory activity of ZNME may be due to the inhibition of free radicals production that act as pro-inflammatory agents in acute and chronic inflammation. So the antioxidant property of ZNME can explain, in part, the mechanism of its anti-inflammatory activity. Present study therefore, substantiates the traditional uses of *Z. nitidum* root in pain and rheumatism in North-East India. Further studies are presently underway to confirm the identity of bioactive principles responsible for these actions by the root of *Z. nitidum*.

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