Enhancement of gut permeation of amoxicillin with *Nigella sativa* seed extract and its phytochemical screening

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**[ABSTRACT]** The seeds of *Nigella sativa* Linn. (Ranunculaceae), commonly known as Black cumin, are predominantly used as carminative, antispasmodic, and stimulant. The main objective of the present study was to evaluate the effect of *N. sativa* seed extract on the permeation of co-infused amoxicillin across the gut wall. The methanolic extract of *N. sativa* improved intestinal permeability of amoxicillin in *in-vitro* experiments in a dose-dependent manner. Two new glycosides, decanyl nigelloic acid diglucoside \[\text{[n-decanyl-3-aldehydic-4-methoxy-5-hydroxy benzoate-5-β-D-glucopyranosyl (2→1)-β-D-glucopyranosyl]}\] and nigel labdienoyl triglucoside \[\text{[homo-labd-5, 9(11)-dien-16-onyl-β-D-glucopyranosyl (2→1)-β-D-glucopyranosyl (2→1)-β-D-glucopyranoside]}\], along with seven known fatty acid glycerides/esters, were isolated from the gut permeation enhancing extract. The structures of these new glycosides were elucidated by detailed spectroscopic analyses.

**[KEY WORDS]** *Nigella sativa*; Everted rat sac; Homolabdane; Amoxicillin; Bioavailability

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**Introduction**

Amoxicillin, a beta-lactam antibiotic, is classified as a class III drug, according to Biopharmaceutics Classification System due to its low permeability [1]. It is conventionally administered as tablet, oral suspension or injection. The drug shows poor oral absorption due to complete ionization under gastrointestinal pH conditions and exhibits very low lipid solubility. In rat plasma profile studies, the drug has revealed low bioavailability [2]. It has also been confirmed by the reports obtained from a regional perfusion study in humans [3]. Among the reasons for poor oral bioavailability of drugs, the intestinal epithelium acts as a prime barrier [4].

*Nigella sativa* Linn. (Ranunculaceae), commonly known as black cumin, is an annual flowering plant and is found mainly in the Mediterranean region. It is indigenous to southwest Asia and is extensively used in the Indian diasporas as spice [5]. It is considered carminative, stimulant, diuretic, emmenagogue, and galactagogue, whereas its oil is applied externally for skin eruptions as antiseptic [6-8]. Fixed oil, volatile oil, thymoquinone, and alkaloids have been reported earlier from the plant [9]. In our previous studies, linoleic acid, oleic acid, margaric acid, cis-11, 14-eicosadienoic acid and stearic acid are found to be the major components of the hexane extract [10]. We have also reported that its fixed oil improves the permeation of carvedilol and amoxicillin [11-12]. The effect is mainly attributed to the presence of fatty acids, such as oleic acid, linoleic acid and stearic acid. In the current study, we evaluated the gut permeation enhancing effect of methanolic extract of *N. sativa* seeds and isolated two new glycosides from the active extract, along with seven known compounds.

**Results and Discussion**

The effect of methanolic extract of *N. sativa* seeds on gut permeation of amoxicillin was investigated using excised rat intestinal segments. The cumulative amount of amoxicillin permeated when used alone and in combination with different concentration of *N. sativa* methanol extracts were calculated and results are presented in Table 1. A dose-dependent in-
crease in the permeation of amoxicillin was observed. The effect was significantly higher with 6 mg \((P < 0.001)\) and 3 mg \((P < 0.05)\) of methanolic extract as compared to the control. As the methanolic extract showed enhancement in amoxicillin permeation in in-vitro studies, it was examined phytochemically by column chromatography over silica gel.

Table 1 Cumulative concentration of amoxicillin permeated in presence of methanolic extract of Nigella sativa seeds in in-vitro study

<table>
<thead>
<tr>
<th>(\tau/\text{min})</th>
<th>Permeated concentration of amoxicillin ((\mu\text{g} \cdot \text{mL}^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control amox (6 mg mL(^{-1}))</td>
</tr>
<tr>
<td>15</td>
<td>7.39 ± 0.07</td>
</tr>
<tr>
<td>30</td>
<td>9.05 ± 0.40</td>
</tr>
<tr>
<td>45</td>
<td>10.52 ± 0.50</td>
</tr>
<tr>
<td>60</td>
<td>13.91 ± 0.88</td>
</tr>
<tr>
<td>75</td>
<td>15.13 ± 0.98</td>
</tr>
<tr>
<td>90</td>
<td>17.53 ± 1.53</td>
</tr>
</tbody>
</table>

Amox, Amoxicillin; NME, Nigella methanath extract. The values represent mean of three observations ± SD; *\(P < 0.05\) and **\(P < 0.001\) compared to control.

Seven known compounds consisted of glyceryl oleyl linoleate (1), glyceryl oleyl dinoleate (2), glyceryl dinoleyleodogolate (3), glyceryl oleyl godolyl linolate (4), glyceryl oleyl stearly hydroxy benzoate (5), glyceryl oleyl stearate (6), and ethyl oleate (7). These fatty acid glycerides or esters were identified on the basis of spectral comparison with related compounds \([13-15]\).

Compound 8 was obtained as pale yellow crystals from chloroform-methanol (3 : 1) eluents. Its FTIR spectrum displayed absorption bands at 3 480, 3 334 cm\(^{-1}\) (hydroxyl group), 1 731 cm\(^{-1}\) (ester group), 1 701 cm\(^{-1}\) (carbonyl group), and 1 520 cm\(^{-1}\) (aromatic moiety), respectively. Its ESI+ mass spectrum exhibited a molecular ion peak at \(m/z\) 804 consistent with the molecular formula \(C_{39}H_{40}O_{19}\) (HR-ESI-MS [M]+ 804.800 5). The \(^1\)H NMR spectrum of 8 displayed a downfield one-proton broad signal at \(\delta\) 9.27 assigned to H-8 aldehydic proton. Two one-proton doublets at \(\delta\) 7.20 and 6.23 \((J = 3.0\) Hz, each) were assigned to H-6 and H-2 aromatic protons, respectively. Three one-proton doublets at \(\delta\) 5.01 \((J = 7.0\) Hz), 4.90 \((J = 7.3\) Hz) and 4.63 \((J = 7.1\) Hz) were attributed correspondingly to H-1’, H-1” and H-1’” anomeric protons. A three-proton broad signal at \(\delta\) 4.22 was accounted to methoxy protons located on the aromatic ring. The remaining sugar protons resonated between \(\delta\) 4.02–3.07. The methylene protons of aliphatic chain appeared between \(\delta\) 2.23–1.01. A three-proton triplet at \(\delta\) 0.59 \((J = 6.5\) Hz) was assigned to Me-10”” primary methyl protons. The \(^{13}\)C NMR spectrum of 8 exhibited signals for aldehydic carbon at \(\delta\) 197.85 (C-8); ester carbon at \(\delta\) 177.50 (C-7); aromatic carbons between \(\delta\) 121.63 and 161.99; and anomic carbons at \(\delta\) 109.31(C-1’), 101.26 (C-1’”), and 98.47 (C-1”’). The remaining sugar carbons resonated between \(\delta\) 60.93–82.51 and primary methyl carbon C-10”” at \(\delta\) 13.05. The presence of C-1’ carbon signal at \(\delta\) 109.31 and C-4’ carbon signal at \(\delta\) 82.51 indicated the furanose form of one of the sugar units. The appearance of C-2’ and C-2”’ signals in the deshielded region at \(\delta\) 74.81 and 79.53, respectively, indicated 2→1 linkages of the sugar units. The HMBC spectrum of 8 displayed cross peaks between aldehydic proton and methoxy carbon, indicating their spatial proximity. The correlations observed between aromatic protons H-2/H-6 and C-7 were also observed (Fig. 1). The spectral data of 8 were compared to the related compounds \([16]\). On the basis of above discussion, the structure of 8 was elucidated as n-decanyl-3-aldehydic-4-methoxy-5-hydroxy benzoate-5-β-D-glucofuranosyl (2→1)-β-D-glucopyranosyl(2→1)-β-D-glucopyranoside and it was named as decanylnigellic acid diglucoside.

Compound 9 was obtained as pale yellow crystalline mass from chloroform-methanol (3 : 1) eluents. Its FTIR spectrum exhibited absorption bands at 3 510, 3 425 cm\(^{-1}\) (hydroxyl group), 1 736 cm\(^{-1}\) (ester group), and 1 641 cm\(^{-1}\) (unsaturation). Its ESI+ mass spectrum displayed a molecular ion peak at \(m/z\) 620 consistent with the molecular formula \(C_{39}H_{40}O_{19}\) (HR-ESI-MS [M]+ 621.520 1). The fragment ion peaks at \(m/z\) 301 [C\(_2\)H\(_3\)O\(_2\)] and 503 [M – 301] due to ester fission along with ion peak at \(m/z\) 203 [C\(_4\)H\(_7\)O] due to C\(_{11,12}\) fission suggested the presence of a labdane unit in 9. The \(^1\)H NMR spectrum of 9 displayed two one-proton multiplets at \(\delta\) 5.42 and 5.34 ascribed to H-6 and H-11 vinylic protons. Three one-proton doublets at \(\delta\) 5.25 \((J = 7.1\) Hz), 5.20 \((J = 7.2\) Hz) and 4.95 \((J = 6.9\) Hz) were ascribed correspondingly to H-1’, H-1” and H-1’” anomeric protons. The remaining sugar protons resonated between \(\delta\) 3.36 and 4.14. Three broad signals at \(\delta\) 0.70, 0.79, and 0.91 and two doublets at \(\delta\) 0.94 \((J = 8.4\) Hz) and 0.97 \((J = 8.1\) Hz), each integrating for three protons, were assigned to Me-17, Me-18, and Me-19 quaternary methyl and Me-20 and Me-21 tertiary methyl protons, respectively. The \(^{13}\)C NMR spectrum of 9 exhibited important signals for ester carbon at \(\delta\) 173.89 (C-16) and vinylcyclic carbon at \(\delta\) 136.59 (C-5), 132.36 (C-6), 143.56 (C-9), and 121.58 (C-11). The anomeric carbons appeared at \(\delta\) 104.05 (C-1’), 102.59 (C-1’”), and 91.77 (C-1”’). The remaining sugar carbons resonated between \(\delta\) 60.75 and 82.44. The appearance of C-2’ and C-2”’ in the deshielded region at
Fig. 1 Structures of compounds 8 and 9 showing key HMBC correlations

δ 82.44 and 79.88 indicated (2→1) linkages of the sugar units. The HMBC spectrum of 9 displayed interactions of H-20 protons with C-9 and of H-21 protons with C-11, respectively. Long range correlation observed between anomeric proton H-1′ and C-16 supported the presence of sugar chain at C-16 (Fig. 1). The spectral data of 9 were compared to the related compounds [17-18]. On the basis of discussion the structure of 9 was characterized as homolabd-5, 9(11)-dien-16-onyl-β-D-glucopyranosyl (2→1)-β-D-glucopyranosyl (2→1)-β-D-glucopyranoside and it was designated as nigelabdienoyl triglucoside.

The fatty acid glycerides (1−6) and ester (7) can be postulated to be responsible for the bioavailability enhancement. Our results were in agreement with earlier reports where a variety of phytoconstituents from alcoholic extract and volatile oil of black cumin have been found to affect the smooth muscle tissues [19-20]. In conclusion, the results from the present study indicated that N. sativa methanolic extract improved the availability of co-infused amoxicillin by increasing its permeation across gut wall. The extract was found to be efficient absorption enhancer and could be the part of bioavailability enhancing systems for various low permeable drugs. Moreover, a new phenolic acid glycoside (8) and a diterpenic glycoside (9) were isolated from the methanolic seed extract.

Material and Methods

Plant materials
Black cumin seeds were collected from Green Earth Products Pvt. Ltd., Delhi, India and identified and authenticated by Dr. H. B. Singh, Head, Raw Materials Herbarium and Museum, National Institute of Science Communications and Information Resources (NISCAIR), Delhi. A voucher specimen with reference number NISCAIR/RHMD/1147/179/01 has been preserved in the herbarium for future reference.

Chemicals
Amoxicillin was obtained as gift sample from Ranbaxy Research Labs, Gurgaon, India. Potassium dihydrogen orthophosphate, sodium hydroxide, acetonitrile (LC-MS grade, assigned purity 99.9%; Lot No. 90525), and ammonium acetate were purchased from Sigma-Aldrich, Munich, Germany. Water used in the entire analysis was prepared in-house with Milli-Q water purification system procured from Millipore (Millipore Corporation, Burlington, MA, USA). All other chemicals used in the present study were of analytical grade from S.D. Fine Chemicals, Mumbai, India.

Extraction
Seeds of N. sativa (1 kg) were coarsely powdered and extracted exhaustively with methanol for 72 h in a Soxhlet apparatus. The methanol extract was then dried under reduced pressure to get 90 g of dark brown mass (9% yield). The residue was stored under refrigeration until used.

Intestinal permeation studies
Preparation of everted rat sac
The study protocol was duly approved by our Institutional Animal Ethics Committee (No. 639/09). Adult male Wistar albino rats weighing 120–150 g were procured from the Central Animal Facility and housed in cages under standard laboratory conditions (10 h dark/14 h light, temperature 20–25 ºC, relative humidity 65%) for seven days. The animals were fasted for 18 h before the experiment. After decapitation, the abdomen of rats was opened by a midline incision. The entire small intestine was removed quickly by cutting across the upper end of duodenum and the lower end of ileum, and stripping the mesentery manually [21]. The small intestine was then washed out with normal saline solution (0.9% NaCl, W/V) using a syringe equipped with blunt end. Intestinal segments (8 ± 2 cm) were everted. After being blot- ted with a piece of filter paper, a glass weight (1 g) was fixed and tied to the end of everted gut segment to make an empty gut sac. This was important to prevent peristaltic muscular contractions, which could otherwise alter the shape and internal volume of the sac.
In-vitro everted rat sac permeation study

The verted rat sac model was established using the Wilson & Wiseman (1954) method with slight modifications, and used for permeation enhancement studies [22]. The empty sac was filled with 1 mL of amoxicillin (6 mg·mL⁻¹) in phosphate buffered solution (PBS) (pH 7.4) using a blunt-end syringe. The loose ligature on the proximal end was tightened. The serosal compartment contained buffer in the sac. The distended sac was placed inside the organ tube of organ bath containing 50 mL of PBS. This gut sac bath was surrounded by a water jacket maintained at 37 ± 0.5 °C. The mucosal fluid compartment content was continuously mixed with air bubbles using an aerator. At predetermined intervals, 5 mL of sample was withdrawn from the organ tube and same volume of fresh buffer was replaced. The concentration of amoxicillin that traversed intestinal surface was monitored spectrophotometrically by recording absorbance at 273 nm [23]. A solution of amoxicillin (6 mg·mL⁻¹) was prepared in PBS (pH 7.4). PBS was prepared by mixing 250 mL of 0.2 mol·L⁻¹ potassium dihydrogen orthophosphate and 393.4 mL of 0.1 mol·L⁻¹ sodium hydroxide, both dissolved in distilled water and final volume made up to one liter. Experiment was repeated with amoxicillin along with 3 and 6 mg of *N. sativa* seed extract separately. All the experiments were conducted in triplicate.

Cumulative concentration of amoxicillin permeated was calculated from the standard plot of amoxicillin in PBS. Amoxicillin (10 mg) was accurately weighed and dissolved in 100 mL of PBS (pH 7.4) to prepare a stock solution 100 µg·mL⁻¹. Different dilutions ranging from 10 to 100 µg·mL⁻¹ of amoxicillin were then prepared by serial dilution method. Absorbance was recorded at 273 nm. PBS was used as blank and absorbance was plotted against concentration. The results of in-vitro permeation study are presented in Table 1.

**Data analysis and statistical analysis**

The results are expressed as means ± standard deviation (SD). Statistical comparisons were performed by a one-way analysis of variance test. When comparison of more than two groups revealed statistically significant differences, the multiple-comparison Tukey’s test was applied in order to find out which groups were different. A probability level of less than 0.05 was considered statistically significant.

**Isolation and characterization of compounds**

The dried extract (70 g) was dissolved in a minimum amount of methanol and adsorbed on silica gel (60−120 mesh) for the preparation of slurry. The air-dried slurry was chromatographed over silica gel column packed in petroleum ether (60−80 °C). The column was initially eluted with chloroform that resulted in the isolation of oily compounds (1−4). Further elution of column with chloroform−methanol (9 : 1) and chloroform−methanol (3 : 1) yielded Compounds 5−7 and 8−9, respectively.

**Table 2** ¹H (500 MHz) and ¹³C NMR (125 MHz) spectral values of compounds 8 and 9

<table>
<thead>
<tr>
<th>Position</th>
<th>¹H NMR (J/ in Hz)</th>
<th>¹³C NMR</th>
<th>Position</th>
<th>¹H NMR (J/ in Hz)</th>
<th>¹³C NMR</th>
</tr>
</thead>
<tbody>
<tr>
<td>¹</td>
<td>5.01 d (7.0)</td>
<td>109.31</td>
<td>9</td>
<td>1.88 m</td>
<td>46.31</td>
</tr>
<tr>
<td>2</td>
<td>4.10 m</td>
<td>74.81</td>
<td>10</td>
<td>–</td>
<td>32.68</td>
</tr>
<tr>
<td>3</td>
<td>3.81 m</td>
<td>70.45</td>
<td>11</td>
<td>5.34 m</td>
<td>121.58</td>
</tr>
<tr>
<td>4</td>
<td>–</td>
<td>82.51</td>
<td>12</td>
<td>2.05 m, 2.03 m</td>
<td>26.49</td>
</tr>
<tr>
<td>5</td>
<td>3.36 d (6.0), 3.34 d (6.0)</td>
<td>65.28</td>
<td>13</td>
<td>1.88 m</td>
<td>25.68</td>
</tr>
<tr>
<td>6</td>
<td>3.20 d (7.5), 3.17 d (7.5)</td>
<td>61.26</td>
<td>14</td>
<td>1.28 m, 1.32 m</td>
<td>23.34</td>
</tr>
<tr>
<td>7</td>
<td>–</td>
<td>79.53</td>
<td>16</td>
<td>–</td>
<td>173.89</td>
</tr>
<tr>
<td>8</td>
<td>9.27 br s</td>
<td>197.85</td>
<td>8</td>
<td>1.88 m</td>
<td>46.31</td>
</tr>
<tr>
<td>9</td>
<td>1.63 m, 1.32 m</td>
<td>31.69</td>
<td>1</td>
<td>–</td>
<td>143.56</td>
</tr>
<tr>
<td>10</td>
<td>–</td>
<td>74.81</td>
<td>11</td>
<td>5.34 m</td>
<td>121.58</td>
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<tr>
<td>11</td>
<td>–</td>
<td>70.45</td>
<td>12</td>
<td>2.05 m, 2.03 m</td>
<td>26.49</td>
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<tr>
<td>12</td>
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<td>25.68</td>
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<tr>
<td>13</td>
<td>1.28 m, 1.32 m</td>
<td>41.70</td>
<td>14</td>
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<tr>
<td>14</td>
<td>–</td>
<td>173.89</td>
<td>15</td>
<td>2.35 d (8.1), 2.32 d (8.1)</td>
<td>41.70</td>
</tr>
<tr>
<td>15</td>
<td>0.70 br s</td>
<td>17.57</td>
<td>16</td>
<td>–</td>
<td>173.89</td>
</tr>
<tr>
<td>16</td>
<td>0.79 br s</td>
<td>16.82</td>
<td>17</td>
<td>0.70 br s</td>
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</tr>
<tr>
<td>17</td>
<td>0.91 br s</td>
<td>16.23</td>
<td>18</td>
<td>0.79 br s</td>
<td>16.82</td>
</tr>
<tr>
<td>18</td>
<td>0.94 d (8.4)</td>
<td>15.35</td>
<td>19</td>
<td>0.91 br s</td>
<td>16.23</td>
</tr>
</tbody>
</table>
Pale yellow crystals, recrystallized from MeOH, 203 mg (0.290%), mp: 70–72 °C, UV λ\text{max} (MeOH): 206, 260 nm (log ε 4.8, 2.5), IR ν\text{max} (KBr): 3 480, 3 334, 2 963, 2 927, 2 853, 1 721, 1 701, 1 520, 1 443, 1 072, 917 cm\(^{-1}\); \(^{1}H\) and \(^{13}C\) NMR (DMSO-d\(_6\)): Table 2, +ve ion ESI MS (M)\(^{+}\): 804 \([M]^{+}\) (C\(_{37}\)H\(_{56}\)O\(_{19}\)) (11.2), 157 (6.3).

Pale yellow crystals, recrystallized from MeOH, 152 mg (0.217%), mp: 176–178 °C, UV λ\text{max} (MeOH): 205, 260 nm (log ε 4.9, 3.8), IR ν\text{max} (KBr): 3 510, 3 425, 3 390, 3 260, 2 926, 2 855, 1 736, 1 641, 1 382, 1 053, 925 cm\(^{-1}\); \(^{1}H\) and \(^{13}C\) NMR (MeOD): Table 2, +ve ion ESI MS m/z (rel. int.): 820 \([M]^{+}\) (C\(_{37}\)H\(_{57}\)O\(_{19}\)) (11.2), 157 (6.3).

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### References


