Antibacterial Properties of Secoisolariciresinol Diglucoside Isolated from Indian Flaxseed Cultivars

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Abstract
Secoisolariciresinol diglucoside (SDG) is an important lignan found in flaxseed and is an emerging source in the functional food area. In the present study, antibacterial properties of SDG extracts from hull, endosperm and flour fractions of Indian flaxseed (Linum usitatissimum) varieties (LVF-01 and GVF-03) were evaluated. The SDG extracts were tested against the six bacterial species Pseudomonas aeruginosa, Staphylococcus aureus, Bacillus subtilis, Agrobacterium tumefaciens, Bacillus cereus, and Escherichia coli. The maximum SDG and phenolic contents were found to be 16.9 and 12.5 mg/g and 3.18 and 2.70 mg/g in hull fractions of LVF-01 and GVF-03 respectively, when compared to flour and endosperm fractions. Among the fractions, the hull fraction of LVF-01 showed maximum activity 31.5 mm at MIC 100 ppm against E. coli, while minimum inhibitory activity was 3.1 mm with MIC at 300 ppm against B. subtilis. Similarly, in the case of GVF-03, maximum activity (31.9 with MIC 150 ppm) of the hull fraction, whereas, its minimum inhibitory activity was (2.3 mm, with MIC 350 ppm) against B. subtilis, when compared to endosperm and flour fractions.

Keywords: Flaxseed, lignans, SDG extracts, phenolics, HPLC, antibacterial properties

Introduction
Flaxseed (Linum usitatissimum) is the most valuable oil seed crop grown in several areas around the world. It is processed for its oil and meal. In flaxseed, the hull or seed coat is tightly adhered to the embryo and it is very difficult to separate unlike many other oilseeds in their pure form without oil extraction. The hull portion is rich in fibre and lignans, whereas, the endosperm is higher in oil and protein content. Flaxseed has gained importance in food industries as a component in designer food, functional food and in value added products because of its high content of lignans, which exert nutraceutical and therapeutic principles (1). Flaxseed is the richest source of phytoestrogen or plant lignan SDG

Fig. 1: Structure of SDG (2, 3-bis [(4-hydroxy-3-methoxyphenyl) methyl] -1, 4 butane-diglucoside) (Rajesha et al. 2008)
and constitutes about 75-800 times higher than vegetarian food sources (2). In addition to high content of SDG; mammalian lignan precursor, flaxseed is also well known for other lignan precursors such as matairesinol relatively in lower level (3, 4). Lignans are an important phytoestrogen with weak estrogenic and anti-estrogenic properties, and possesses diverse bioactivities. Epidemiological studies have reported the chemo preventive effects of lignans on tumors of colon, skin and mammary glands (5). SDG exhibits a wide range of health promoting activities, which is effective against the onset of various sort of cancers such as breast, colon and prostate (6, 7). The consumption of flaxseed based diet by rats caused protective effects against cardiovascular diseases such as reduction in the level of LDL cholesterol and aortic atherosclerosis (8). Flaxseed is well known for its hydroxyl radical scavenging activity of SDG and antioxidant activities (9) in addition to ED and EL in vitro (10). Lignans also exert antibacterial and cytotoxic activities, antitumor and antivirus etc., (11). The production of mammalian lignans ED and EL after flaxseed ingestion have been shown to inhibit aromatase activity and stimulate production of sex hormone binding globulin (SHBG), which is hypothesized to the reduction of endogenous estrogen level and lengthening of the estrous cycle in in vitro and animal models (12, 13).

There is growing awareness of flaxseed as a source of food and for several therapeutic purposes. Further, there is lack of information on antibacterial properties of SDG isolated from different fractions obtained upon its dehulling or milling process. Hence, the present study was under taken to evaluate the antibacterial properties of SDG isolated from hull, endosperm and flour against important some pathogenic bacteria.

**Materials and Methods**

**Chemicals**

All the solvents and chemicals used for the experiment were of analytical grade obtained by Sigma Chemicals Co., St. Louis, MO, USA. Solvents used for HPLC were of HPLC grade and purchased from Ranbaxy fine chemicals Ltd. Mumbai, India

**Flaxseed**

Two flaxseed cultivars, grown at two locations, Ranebennur and Gadag, North Karnataka, India were purchased from the local market. The University of Agricultural Sciences, Hebbal, Bangalore, Karnataka, India authenticated the seeds. The specimen samples of seeds LVF-01 and GVF-03 were preserved for analysis. Flaxseeds were processed by the combination of conditioning, de-hulling, sieving and aspiration. The dehulling of the seed was carried out using Kisan Krishi Yantra Udyog, Kanpur, India situated at Department of Grain Science and Technology, CFTRI, Mysore, India. The fractions such as hull, endosperm and flour were obtained after dehulling process.

**Extraction of SDG from flaxseed**

The extracts of SDG were prepared by the Klosterman method described by Rickard et al., (1) from flaxseed fractions such as hull, endosperm and flour obtained upon dehulling process.

**High performance liquid chromatography (HPLC) analysis of SDG in flaxseed fractions**

High performance liquid chromatographic analyses were carried out and the SDG peaks were identified and quantified by comparison with those of the SDG standards, and its amount were also calculated as reported in our recent study (14).
Bacterial strains and culture conditions

The antibacterial activity was tested against *Staphylococcus aureus* (FRI 722), *Bacillus cereus* (F 4433), *Escherichia coli* (D 21) were obtained as generous gift from Dr. E. Notermans, National Institute of Public Health, Netherlands, Dr. J. M. Kramer, Central Public Health Laboratory, United Kingdom and Dr. M. A. Linggood, Unilever Research, United Kingdom, respectively. The strains of *Pseudomonas aeruginosa*, *Bacillus subtilis*, and *Agrobacterium tumefaciens* were obtained from Food Microbiology Department, CFTRI, Mysore, India. (15). All test organisms were maintained on nutrient agar slants (Hi Media chemicals, India). Cultures of *S. aureus* (FRI 722), *B. cereus* (F 4433), *E. coli* (D 21), *P. aerugenosa* (CFR 1704) were grown in brain heart infusion broth (Hi Media, India) for 18h at 37 °C and appropriate cell dilutions were prepared in 0.85 % NaCl to obtain counts of 10^2 and 10^3/ml (16). The respective bacterial counts were determined by surface plating on Baird-Parker agar for *S. aureus*, MacConkey agar for *E. coli* and *Pseudomonas* agar for *P. aeruginosa* (Hi Media, India). Cell suspensions of *Bacillus* species were prepared following the method of Rappaport and Goepfert (17) and cell dilutions were determined by surface plating on Polymyxin Pyruvate Egg yolk Mannitol Bromothymol blue agar (PEMBA) (Hi Media, India).

Determination of total phenolic compounds in SDG extracts

The concentration of total phenolic compounds in the extracts was determined according to the method of Taga et al., (18) and expressed as caffeic acid equivalents. In brief, samples and standards were prepared in acidified (3 g/l HCl) methanol/water (60:40 v/v) and 100 µl of each were added separately to 2 ml of 2% Na2CO3. After 5 min, 100 µl of 50% Folin–Ciocalteu reagent was added and the mixture was allowed to stand at room temperature for 30 min. Absorbance was measured at 750 nm using spectrophotometer (Shimadzu 160A). The blank consisted of all reagents and solvents without sample or standard. The standard caffeic acid was prepared at concentrations of 10-100 µg/ml. The phenolic concentration was determined by comparison with the standards.

In vitro screening for antibacterial activity of SDG extracts

Agar-well diffusion assay

The antimicrobial activity was measured by agar well diffusion assay method (19). Extracts dissolved in ethanol (5 mg/ml) was used for the assay. About 75 µl of the sample was placed in the wells and allowed to diffuse for 2 h. Plates were incubated at 37 °C for 48 h and the activity was determined by measuring the distance of inhibition zones. Ethanol and DMSO alone were used as a control and amoxycillin as a positive control. The assay was carried out in triplicate.

Minimum inhibitory concentration (MIC)

The MIC was determined by the modified method developed by Dufour et al., (20) and Gary et al., (21). Different concentrations (50 ppm to 300 ppm) of test sample and 100 µl of the bacterial suspension (10^5 CFU/ml) was placed aseptically in 10 ml of nutrient broth and incubated for 24 h at 37 °C. The growth was observed both visually and by measuring O.D. at 600 nm at regular intervals followed by plating with nutrient agar. The lowest concentration of test sample showing no visible growth was recorded as the minimum inhibitory concentration. The sample tubes were maintained for each concentration of test sample and the readings were plotted against O.D at 600 nm as growth curves.
Statistical analysis

The data from three replicates were processed by one-way ANOVA using the least significant test to determine the level of significance at $P \leq 0.05$.

Results and Discussion

SDG contents of hull, endosperm and flour fractions of flaxseed

SDG contents were measured in different fractions of both varieties of flaxseeds. The HPLC chromatograms showed the presence of SDG in all the fractions of flaxseed as one of the major lignan among the other lignans, which has shown maximum absorbance at 280 nm and the retention time for SDG was found to be 29-30 min as shown in Figures 1 and 3. In both the varieties, hull fractions showed higher SDG content (16.9 ± 1.25 and 12.5 ± 1.18 mg/g) followed by flour and endosperm fractions. The SDG content of hull and flour fractions were higher by 14 and 5-fold in LVF-01 variety and 20 and 8-fold higher content in GVF-03 variety respectively, when compared to endosperm fraction. The data are presented in the Table 1.

Table 1. Phenolic and SDG contents of hull, endosperm and flour fractions of LVF – 01 and GVF – 03 varieties.

<table>
<thead>
<tr>
<th>Fractions</th>
<th>Flaxseed varieties</th>
<th>Total phenolics (mg/g)</th>
<th>SDG content (mg/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hull</td>
<td>LVF-01</td>
<td>3.18 ± 0.56</td>
<td>16.9 ± 1.25</td>
</tr>
<tr>
<td></td>
<td>GVF-03</td>
<td>2.70 ± 0.74</td>
<td>12.5 ± 1.18</td>
</tr>
<tr>
<td>Endosperm</td>
<td>LVF-01</td>
<td>0.54 ± 0.08</td>
<td>1.2 ± 0.92</td>
</tr>
<tr>
<td></td>
<td>GVF-03</td>
<td>0.22 ± 0.02</td>
<td>0.6 ± 0.03</td>
</tr>
<tr>
<td>Flour</td>
<td>LVF-01</td>
<td>1.34 ± 0.65</td>
<td>5.8 ± 1.42</td>
</tr>
<tr>
<td></td>
<td>GVF-03</td>
<td>0.80 ± 0.02</td>
<td>4.6 ± 1.21</td>
</tr>
</tbody>
</table>

Phenolic contents of hull, endosperm and flour fractions of flaxseed

The content of phenolic compounds in all the fractions of both the flaxseed varieties were estimated (Table 1). The total phenolics of different fractions were found to be 3.18, 0.54 and 1.34 (LVF-01) and 2.70, 0.22 and 0.80 mg/g (GVF-03) in hull, endosperm and flour respectively. Both the varieties showed higher phenolic contents in hull fractions compared to that of the endosperm and flour fractions. The endosperm fraction had the least phenolic content in both the varieties. Total phenolics content in LVF-01 variety was 5.06 mg/g and 62, 11 and 27% was recovered in hull, endosperm and flour respectively.

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fractions respectively, whereas, the phenolics content in GVF-03 variety was 3.72 mg/g and 72, 6 and 22% was recovered in hull, endosperm and flour fractions respectively. When compared to GVF-03 variety, LVF-01 variety had more total phenolics content (36%). The results showed that hull fraction of LVF-01 and GVF-03 contained a higher amount of phenolics than the other two fractions.

**Antibacterial properties of SDG extracts of hull, endosperm and flour fractions of flaxseed**

The extracts of SDG from different fractions of both LVF-01 and GVF-03 were evaluated for their antibacterial activity as shown in Table 2. The varieties, LVF-01 and GVF-03 were specifically selected for antibacterial properties, because of their wide cultivation and widespread use at lower levels as food substitutes in Northern Karnataka. The evaluation of the antibacterial activity of the SDG extracts against bacteria was carried out by agar well diffusion assay method. The SDG of the flax seed fractions of both varieties exhibited antibacterial activity against all tested bacterial strains and showed various degrees of inhibition against them. The SDG from hull fractions of LVF-01 showed maximum

**Fig. 2:** HPLC chromatograms of SDG extracts of hull (1), endosperm (2), flour (3) and standard (4) of LVF-01 variety.

**Fig. 3:** HPLC chromatograms of SDG extracts of hull (1), endosperm (2), flour (3) and standard (4) of GVF-03 variety.
activity (31.5 mm) against \textit{E. coli}, while it was minimum (9.7 mm) against \textit{B. cereus}. Similarly, endosperm SDG exhibited maximum (14.87 mm) activity against \textit{S. aureus} and minimum activity (3.13 mm) against \textit{B. subtilis}. Flour fraction SDG exhibited maximum and minimum activity (22 and 6.83 mm) against \textit{E. coli} and \textit{B. cereus} respectively (Table 2). On the other hand, hull fraction of GVF-03 showed most pronounced activity with inhibition zones of 31.97 mm and minimum activity with 8.7 mm against \textit{E. coli} and \textit{B. cereus} respectively. Similarly, maximum and minimum activities (13.43 and 2.33 mm) for \textit{S. aureus} and \textit{B. subtilis} was observed by SDG from endosperm fraction and also flour-SDG showed maximum (21.57 mm) activity against \textit{E. coli} and minimum activity (7.37 mm) against \textit{B. cereus}.

### Table 2. Antibacterial activity of SDG isolated from different verities (LVF-01 and GVF-03) of flaxseed against bacteria.

<table>
<thead>
<tr>
<th>Microorganisms</th>
<th>LVF - 01</th>
<th></th>
<th></th>
<th>GVF - 03</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Hull</td>
<td>Endosperm</td>
<td>Flour</td>
<td>Hull</td>
<td>Endosperm</td>
<td>Flour</td>
</tr>
<tr>
<td>\textit{P. aeruginosa}</td>
<td>24.7 ± 2.3</td>
<td>6.4 ± 0.4</td>
<td>14.0 ± 0.2</td>
<td>22.5 ± 1.4</td>
<td>4.4 ± 0.3</td>
<td>13.2 ± 1.6</td>
</tr>
<tr>
<td>\textit{S. aureus}</td>
<td>31.5 ± 3.0</td>
<td>14.8 ± 1.0</td>
<td>18.3 ± 1.9</td>
<td>31.7 ± 2.1</td>
<td>13.4 ± 1.3</td>
<td>18.3 ± 2.3</td>
</tr>
<tr>
<td>\textit{B. subtilis}</td>
<td>23.4 ± 1.9</td>
<td>3.1 ± 0.2</td>
<td>16.4 ±1.2</td>
<td>20.7 ± 1.3</td>
<td>2.3 ± 0.2</td>
<td>15.9 ± 1.8</td>
</tr>
<tr>
<td>\textit{A. tumefaciens}</td>
<td>30.3 ± 2.0</td>
<td>10.4 ± 1.2</td>
<td>18.0 ± 1.7</td>
<td>31.2 ± 2.2</td>
<td>8.9 ± 1.0</td>
<td>17.0 ± 1.5</td>
</tr>
<tr>
<td>\textit{B. cereus}</td>
<td>9.7 ± 1.3</td>
<td>7.8 ± 1.9</td>
<td>6.8 ±0.4</td>
<td>8.7 ± 1.3</td>
<td>7.6 ± 1.8</td>
<td>7.3 ± 0.9</td>
</tr>
<tr>
<td>\textit{E. coli}</td>
<td>34.0 ± 2.8</td>
<td>14.0 ± 1.6</td>
<td>22.0 ± 1.6</td>
<td>31.9± 2.4</td>
<td>12.6 ± 1.5</td>
<td>21.5 ± 2.5</td>
</tr>
</tbody>
</table>

Each value represents mean of three different observations ± S.D.

**MIC values for SDG extracts**

The MIC values of SDG fractions of hull, endosperm and flour of LVF-01 ranged from 100 to 300 ppm (Table 3). SDG fraction of hull was very effective against \textit{E. coli} with MIC of 100 ppm and it also inhibited the growth of \textit{S. aureus} and \textit{A. tumefaciens} at 150 ppm. \textit{P. aeruginosa} and \textit{B. cereus} were completely inhibited by SDG fraction of hull at 200 and 300 ppm respectively. SDG extract of endosperm showed inhibitory activity against \textit{S. aureus}, \textit{A. tumefaciens} and \textit{E. coli} with MIC of 250 ppm and \textit{P. aeruginosa}, \textit{B. cereus} and \textit{B. subtilis} at 300 ppm. SDG extract of flour fraction showed inhibition against \textit{E. coli} with 200 ppm and \textit{S. aureus}, \textit{A. tumefaciens} at 200 ppm.

The MIC values of SDG fractions of GVF-03 ranged from 150 to 300 ppm (Table 3). SDG extract of hull showed inhibition against \textit{S. aureus}, \textit{A. tumefaciens} and \textit{E. coli} with MIC of 150 ppm and also it inhibited the growth of \textit{P. aeruginosa} \textit{B. subtilis} at 200 ppm. SDG extract of endosperm fraction showed inhibitory activity against \textit{S. aureus} and \textit{E. coli} with 250 ppm. Similarly, in the case of SDG extract of flour fraction showed inhibitory activity against \textit{E. coli}
at 200 ppm. *S. aureus, B. subtilis, B. cereus* were completely inhibited by SDG extract of flour fraction at 250 and *P. aeruginosa* with MIC of 300 ppm. All the SDG extracts of flaxseed fractions exhibited varied degrees of antibacterial activity. SDG extract of hull fraction of LVF-01 showed higher activity when compared to other fractions.

**Table 3.** MIC values for SDG extracts from different fractions of flaxseed against bacteria.

<table>
<thead>
<tr>
<th>Microorganisms</th>
<th>MIC (ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>LVF - 01 Hull</td>
</tr>
<tr>
<td><em>P. aeruginosa</em></td>
<td>200</td>
</tr>
<tr>
<td><em>S. aureus</em></td>
<td>150</td>
</tr>
<tr>
<td><em>B. subtilis</em></td>
<td>200</td>
</tr>
<tr>
<td><em>A. tumefaciens</em></td>
<td>150</td>
</tr>
<tr>
<td><em>B. cereus</em></td>
<td>300</td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td>100</td>
</tr>
</tbody>
</table>

The present study evaluated the antibacterial properties of SDG isolated from different fractions such as hull, endosperm and flour of two Indian flax seed cultivars against important pathogenic bacteria. The SDG extract of hull of LVF-01 and GVF-03 showed higher activities, when compared to endosperm and flour fractions. SDG extracts of hull of both LVF-01 and GVF-03 showed inhibitory activity against *E. coli, S. aureus, A. tumefaciens* SDG extracts of endosperm fraction of LVF-01 and GVF-03 exhibited inhibitory activity against *S. aureus, A. tumefaciens* and *E. coli*. SDG isolated from flour fractions (LVF-01 and GVF-03) also showed inhibitory activity against *E. coli*. In general, among the investigated extracts of SDG, the extracts of hull of both varieties exhibited highest antibacterial activity than the other two fractions. The results show that the zone of inhibition is a practical approach for screening different concentrations of potential antimicrobial substances. The activities of the SDG extracts of different fractions of flaxseed against bacteria may be indicative to the broad spectrum antibiotic compounds. The differences in antibacterial activity among all the extracts may be correlated with varied quantity of bioactive compounds and phenolics in particular. At low concentration, phenolics are reported to affect enzyme activity, especially of those enzymes associated with energy production while at greater concentrations, they cause protein denaturation. In addition, effect of phenol and fatty acids on microbial growth could be the result of the ability of these compounds to alter microbial cell permeability, permitting the loss of macro-molecules from the interior and could also interact with membrane proteins causing a deformation in their structure and functionality as well as affecting cellular activity as reported by Mundt *et al.* (22).

The spectrum of activity of SDG isolated from different fractions of flaxseed of both...
varieties were active against *E. coli*, and virtually showed less activity against *B. cereus* and *B. subtilis*. In some cases, all the three extracts of the same species had antimicrobial activity against the same microorganism. For instance, the three extracts of LVF-01 were active against *E. coli*. This possibly means that the compound responsible for the antimicrobial activity was present in each extract at a different concentration. Added to this, different results concerning the antibacterial activity might be due to different geographic sources of and types of flaxseed cultivars used. Thus, the difference in the antimicrobial activity of the isolated compounds against gram-positive and gram-negative bacteria may of our study regarding the antibacterial property of SDG extracts indicate that this could be used against the most common pathogens. However, the SDG extracts isolated from different fractions such as hull, endosperm and flour must be studied in animal models to determine their efficacy *in vivo* and possible toxicity, and to elucidate their mechanisms of action.

**Conclusion**

In the present study, it is concluded that the SDG extracts of hull, endosperm and flour from two Indian flaxseed verities are having potential antibacterial activity against pathogens. Among the fractions, the hull fractions were showing higher antibacterial activity when compared to other fractions. The antibacterial effect of SDG of hull and endosperm of flaxseed against clinically important pathogenic bacteria can be a preferred supplement to its known health benefits as antibacterial agents and usage in food system. Further investigations are in progress to study the biological activities of these fractions. Therefore, the different fractions of flaxseed may be recognized as a contributing factor in the preparation of such a type of human health foods as well as others. Further more, careful investigations are required to elucidate the mechanism(s) of action of these compounds. The presence of significant amount of SDG and other lignans in flaxseed, may therefore explain the frequent use of it in a variety of Indian medicinal preparations.

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**References**

Antibacterial properties of SDG


