Antioxidant and antimutagenic properties of calcium sennosides in γ-Irradiated human blood cultures

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Abstract

Background: Calcium sennosides are the main active metabolites of sennas, which have a powerful interest to phytochemical and pharmacological research, due to their brilliant medicinal values. It is well known in folk medicine for their laxative and purgative uses. Materials and Methods: This experiment aimed to assess cytogenetic (micronucleus assay and chromosomal aberration study) and biochemical effects of calcium sennosides at a working dose (24 or 48 mg/ml) on suppressing radiation hazards in human blood cultures. Biochemical investigations include superoxide dismutase (SOD), catalase (CAT), tumor necrosis factor-α (TNF-α), interleukin-8 (IL-8), cyclooxygenase-2 (COX-2) and lactate dehydrogenase (LDH) levels. The treatment periods were 48 and 72 hrs post-irradiation at a dose of 3 Gy. Triple blood cultures for each blood sample were set up. Results: Ionizing radiation exposure induced significant increase in micronuclei (MN) frequencies in both mono- and bi-nucleated cells, and all types of chromosome aberrations, beside significant decrease in SOD and CAT activities. While TNF-α, IL-8, COX-2 and LDH levels were significantly increased after irradiation. Treatment with calcium sennosides exhibited decreased of micronuclei and chromosome aberration numbers and enhancement in the level of SOD and CAT activities. In addition, a significant amelioration in IL-8, TNF-α, COX-2 levels and LDH activity were scored. Conclusion: Present results revealed the antimutagenic and the anti-inflammatory effects of sennosides against oxidative stress induced by γ-irradiation.

Keywords: Calcium sennosides, γ-rays, pro-inflammatory cytokines, micronucleus, chromosomal aberration.

Introduction

Exposure to ionizing radiation is an integral part of our lives in different ways, such as medical, environmental, and/or even accidental. Thus, accepting of the mechanisms by which radiation toxicity develops is crucial to statement acute and chronic health problems that occurs following ionizing radiation exposure. Direct exposure to ionizing radiation-induced free radicals is totally recognized as significant contributors to early and late effects of ionizing radiation. This includes, but is not limited to, inflammation, cytotoxicity and genotoxicity. Damage to the critical biomolecules leading to unfavorable long-term alterations in metabolic redox homeostasis following ionizing radiation exposure has been the focus of various independent investigations over last several decades. The first variety of senna plants were growing on the Nile River banks in Egypt and Sudan, while the second type is widely cultivated in southern and eastern parts of India. Senna plant is a small shrub classified as casual piniaceae family belongs to two genus of Cassia C senna, known as Alexandrion senna,
Angustifolia C senna that named Tinnevelly senna (2). Over the past 6 decades, a large number of aminothiols, natural occurring products, immunomodulators and so on were investigated for their radioprotective potential (3).

The biological dosimetry utilizing dicentric chromosomes examination in human lymphocytes is a well-known method accomplished since long ago together with physical dosimetry for radiation dose assessment in potentially overexposed people as well as for supposed exposures to estimate risk of health effects. The frequency of micronuclei is also commonly used as a cytogenetic biomarker. Another cytogenetic endpoint, cytokinesis-block micronucleus assay, is considered to be simple in terms of scoring criteria as a reliable and sensitive cytogenetic biomarker (4). Moreover, several researches try to manipulate different effects of sennosides such as: protective antioxidant properties, immune stimulating potency in addition to anti-inflammatory strength and its health benefits (5, 6).

In addition, SOD and CAT enzymes involved in oxidative stress have recently been used to monitor the development and extent of oxidative stress damage (7). On the other hand, IL-8 and TNF-α were categorized as pro-inflammatory cytokines predominantly produced by activated macrophages. Pro-inflammatory cytokines could explain most of the local and systemic components of acute and chronic inflammatory diseases. In particular IL-8 is a potent chemotactic agent for neutrophils and induces their lysosomal release (8).

COX known as prostaglandin synthase, is a membrane-bound enzyme responsible for oxidation of arachidonic acid to prostaglandins with two isoforms Cox-1 and Cox-2 had been identified. COX-1 enzyme is constitutively expressed and regulates a number of protection functions such as vascular hemostasis and gastro protection, whereas at the sites of inflammation COX-2 is inducible by number of mediators such as growth factors, cytokines and endotoxins (9). Ionizing radiation was established to induce LDH, lactate production, and extracellular acidification in human and animal tissue in a dose dependent manner (10). The objective of the current study was to evaluate the radio-protective role of different doses of sennosides in irradiated human blood cultures.

**MATERIALS AND METHODS**

**Chemicals**

The chemicals for the blood cultures were purchased from GIBCO-BRL, USA. FA, cytochalasin-B, heat-inactivated foetal calf serum (FCS) and other chemicals and solvents were purchased from Sigma/ Aldrich Chemical Co., St. Louis, USA. Calcium sennosides was purchased from EL-NILE CO. for Pharmaceutical and chemical industries, Egypt, which is available in the form of tablets with the trade name (Senna Lax). Each tablet contained 12 mg of calcium sennosides. The contents of the tablet were dissolved in distilled water and withdrawn by a syringe.

**Blood sampling**

In the purpose of avoiding possible inter-individual variability in response to treatments, blood samples were obtained from equivalent three healthy females (average age 35 years and non-smokers) who offered an informed permission for contribution in this study. Donors were chosen carefully according to International Programme on Chemical Safety guidelines for the monitoring of genotoxic effects of carcinogens in humans (11). Venous blood samples were collected under sanitary conditions in heparinised vacationer test tubes (V= 5 ml, Becton Dickinson, USA) containing lithium heparin to avoid blood coagulation. The study was approved by the Central Scientific Publishing Committee, Egyptian Atomic Energy Authority, RF-156, 5-2016.

**Experimental design**

For each donor sample, blood was divided into 6 groups: in each group 3 samples were processed (n=3). Experimental groups were intended as the following: Group 1: control blood, groups 2 & 3: in these groups blood cultures were
treated with two doses of calcium sennosides (24 & 48 mg/ml) respectively. Group 4: blood samples were exposed to 3Gy of γ-rays. Finally 5 & 6 groups; they combined both treatment, radiation exposure (as in group 4) and calcium sinusitis treatment (as in groups 2 & 3). Two blood cultures were carried on: the first one was cultured for 48 hrs for chromosome aberration analysis and biochemical investigations. The second part was cultured for 72 hrs for micronucleus test and same biochemical parameters.

**Blood culture**
To 0.5 ml of the whole blood, 5ml culture medium (RPMI-1640) supplemented with 20% FCS, 200 m M l-glutamine, penicillin 100 units/ml and streptomycin 100μg/ml were added in 15 ml conical tubes. Phytohaemagglutinin-M with dose 0.2 ml was added to the culture to initiate cell division. Then, cells were incubated at 37°C.

**Irradiation source**
γ-rays were delivered to cell cultures through Canadian 137Cs source that belongs to National centre of Radiation Research and Technology, Atomic Energy Authority, EGYPT. The adjusted dose was 3 Gy. The dose rate was 0.42 Gy/min. The samples were kept at 37°C immediately after irradiation till the treatment periods in the cultures with calcium sennosides started.

**Cytogenetic analysis:**
**Cytokinesis-blocked micronucleus assay (CBMN)**
CBMN was assayed by blocking cells at the cytokinesis stage by the method of Fenech (12). In each sample, a total of 1000 mono- and binucleated cells (Mono. and BN. Cells) were scored and the frequencies of cells with one, two, three micronuclei (MN) were recorded.

**Chromosomal aberration analysis**
The structural chromosome aberration analysis was performed according to current International Atomic Energy Authority (IAEA) guidelines (13). A total number of 100 metaphases were scored for each sample. Each type of aberrations, as well as the percentage of aberrant cells per subject was evaluated.

**Biochemical estimations**

**Determination of SOD activity:**
In SOD activity determination Xanthine-xanthine oxidase used to generate O2− and nitroblue tetrazolium (NBT) decline was used as an indicator of O2•− production. SOD will compete with NBT for O2•−; the percent inhibition of NBT reduction is a measure of the amount of existing SOD. SOD activity was measured in both 48 & 72 hrs cell culture supernatants. For each tube 0.5 cacodylic buffer 0.1ml of triton X100, 0.25 ml of NBT and 1 ml of cell culture supernatants were mixed, then the mixture was incubated for 5 min at 37oC then start the reaction by adding 0.1 ml of pyrogallol, after 5 min add 0.3 ml stop solution to stop the reaction. 1.0 ml of distilled water was added instead of cell culture supernatant in blank. Finally at 540 nm optical density was measured spectrophotometrically. This quantitative method was carried on according to (14).

**Determination of Catalase activity:**
Catalase activities were measured in cell culture supernatant, according to Johansson and Borg (15). Reaction of catalase enzyme with methanol in the presence of an optimal concentration of hydrogen peroxide was the idea of work. 400μl of cell culture supernatant, 50μl of methanol, 50 μl of KH2PO4/NaOH and 10μl hydrogen peroxide were mixed. Mixture was incubated in shaking water bath (WB) for 20 min at 20 °C. 50μl of potassium hydroxide and 100μl of purpald solution was added to the mixture. Incubated again in WB for 10 min. at 20 °C then 50 μl of potassium periodate was added to the mixture then centrifuged at 9500 g for 10 min. Colour compound was developed from the reaction of purpald and formaldehyde was measured spectrophotometrically at 550 nm.

**Pro-inflammatory enzymes: (Cox-2 & LDH):**
In the present study both COX-2 and LDH activities were measured in cell cultures supernatant via Enzyme-Linked Immunosorbent

Assay (ELISA) kit purchased from MYBioSource using double sandwich ELISA technique. The pre-coated antibody is Human COX-2 as well as LDH monoclonal antibody and the detecting antibody is polyclonal antibody with biotin labelled. Samples and biotin labelling antibody were added into ELISA plate wells and carefully washed out with phosphate buffer saline (PBS). Then Avidin-peroxidase conjugates were added to wells in order. 3,3',5,5'-Tetramethylbenzidine (TMB) substrate was used for colouring after reactant carefully washed out by PBS. TMB changed in colour into blue in peroxidase catalytic then finally into yellow under the action of acid. The colour depth and the testing factors in samples were positively correlated.

**Pro-inflammatory Cytokines (IL-8 & TNF-α):**

Human IL-8 and TNF-α were measured using solid phase sandwich ELISA from Abcam. Abcam's IL-8 as well as TNF-α in vitro Simple Step ELISA kit was used for the quantitative measurement of IL-8 & TNF-α protein in Human cell culture supernatant.

**Statistical analysis**

Data are presented as distribution analysis, means ± S.E. and analysed using two ways analysis of variance "F" test according to Abramowitz and Stegun (16). The level of statistical significance was $P < 0.05$.

**RESULTS**

Table 1 shows significantly increments of cells with MN frequencies (1, 2 & 3) in human lymphocytes culture exposed to 3 Gy of γ-rays, compared to control and calcium sennosides groups. Binucleated cell frequencies in irradiated group were less than that of control and calcium sennosides groups. Significant improvements occurred in all percentages of micronuclei and binucleated cells after blood culture in calcium sennosides treated media.

The most representative aberration types in chromosome aberration analysis, after irradiation is breaks, fragments and dicentrics. Figure 1 shows increments of all aberration types especially, for fragments and dicentrics, after irradiation comparing with control and calcium sennosides groups. Calcium sennosides treatments ameliorated the recorded aberration numbers.

Table 2 shows marked inhibition in both SOD & CAT activities after ionizing radiation exposure in two periods of culture (48 & 72 hrs) when compared with their corresponding control groups, while treating cultures with calcium sennosides revealed marked amelioration in both enzyme activities. SOD levels exhibited a significant increase in culture 72 hrs only when compared with that of radiation group. Also, results showed that CAT activity recorded a significant increase in both treated doses of calcium sennosides (24 & 48 mg/ml) in the two culture periods (48 & 72 hrs).

On the other hand, radiation induced significant elevation in COX-2 enzyme activities in the two cultures (48 & 72 hrs) as 1.5 folds than that recorded in control group. Calcium sennosides treatments (24 & 48 mg/ml) after irradiation tends to normalize COX-2 activities with significant change from irradiated group as illustrated in figure 2.

Figure 3 shows that LDH levels exhibited a significant increase in the radiation group in both cultures 48 & 72 hrs when compared with their corresponding control groups. A marked decrease in LDH levels were detected after treatment of irradiated cultures (48 & 72 hrs.) with calcium sennosides (24 & 48 mg/ml) than that detected in irradiated cultures.

Figure 4 shows bar charts that represents the control TNF-α in human blood culture at 48 hrs as (13%) while the irradiated group recorded an increase in TNF-α concentration about (13%) more than control while calcium sennosides (24 mg/ml) treated group recorded only (7%) increase in its level & calcium sennosides (48 mg/ml) recorded amelioration in TNF-α concentration as increased only by (2%) from control group.

Furthermore, results obtained in human blood culture at 72 hrs showed that TNF-α control levels recorded (15%). Whereas in irradiated group, TNF-α level was increased by (9%) more...
than control value, while treatment with calcium sennosides at doses of 24 & 48 mg/ml showed increase in its level by 3% more than that of control group.

IL-8 levels represent 16% in culture 24 & 48 hrs respectively. In irradiated groups, IL-8 levels elevated at 5% and 8% in the culture of 48 and 72 hrs respectively, more than that recorded in control group. IL-8 level declined after treatment of irradiated blood cultures (48 and 72 hrs) with calcium sennosides 24 &48 mg/ml to reach levels that of control group (figure 5).

### Table 1. CBMN test in human blood culture in vitro treated with sennosides (24 & 48 mg/ml) after 3Gy γ-rays exposure.

<table>
<thead>
<tr>
<th>Group</th>
<th>Control group</th>
<th>Sennosides (24 mg/ml) group</th>
<th>Sennosides (48 mg/ml) group</th>
<th>Radiation group</th>
<th>Rad. &amp; Sennosides (24 mg/ml) group</th>
<th>Rad. &amp; Sennosides (48 mg/ml) group</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mono.cells+ 0 MN</td>
<td>404.3 ± 9.34</td>
<td>418.3 ± 6.01</td>
<td>417.7 ± 4.34</td>
<td>432.3 ± 4.34</td>
<td>422.0 ± 2.52</td>
<td>427.33 ± 3.85</td>
</tr>
<tr>
<td>Mono.cells+1MN</td>
<td>2.0 ± 0.58</td>
<td>2.0 ± 0.58</td>
<td>2.0 ± 0.5</td>
<td>22.3 ± 1.45</td>
<td>13.3 ± 1.20</td>
<td>14.3 ± 0.88 ab,c,d</td>
</tr>
<tr>
<td>Mono.cells+2 MN</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
<td>11.3 ± 0.88</td>
<td>5.3 ± 0.88 abcde</td>
<td>8.0 ± 0.58 abcde</td>
</tr>
<tr>
<td>Mono.cells+3 MN</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
<td>4.3 ± 0.33</td>
<td>1.6 ± 0.88 abcde</td>
<td>2.0 ± 1.0 abcde</td>
</tr>
<tr>
<td>Total Mono. cells</td>
<td>406.3 ± 9.15</td>
<td>420.3 ± 5.46</td>
<td>419.6 ± 4.67</td>
<td>468.3 ± 5.79</td>
<td>442.3 ± 3.18 abcde</td>
<td>451.6 ± 3.18 abcde</td>
</tr>
<tr>
<td>BN+ 0 MN</td>
<td>591.0 ± 9.08</td>
<td>576.7 ± 5.46</td>
<td>577.0 ± 4.93</td>
<td>526.3 ± 34.51</td>
<td>535.3 ± 3.53 abcde</td>
<td>524.0 ± 2.52 abcde</td>
</tr>
<tr>
<td>BN+ 1 MN</td>
<td>2.6 ± 0.33</td>
<td>3.0 ± 0.0</td>
<td>3.3 ± 0.33</td>
<td>27.3 ± 1.77</td>
<td>17.3 ± 1.77 abcde</td>
<td>18.3 ± 1.20 abcde</td>
</tr>
<tr>
<td>BN+ 2 MN</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
<td>8.6 ± 0.88</td>
<td>4.3 ± 0.33 abcde</td>
<td>5.0 ± 0.58 abcde</td>
</tr>
<tr>
<td>BN+ 3 MN</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
<td>2.7 ± 0.33</td>
<td>1.0 ± 0.58</td>
<td>1.67 ± 0.33 abcde</td>
</tr>
<tr>
<td>Total BN</td>
<td>593.7 ± 9.15</td>
<td>579.7 ± 5.46</td>
<td>580.3 ± 4.67</td>
<td>531.7 ± 5.77</td>
<td>557.7 ± 3.18 abcde</td>
<td>548.3 ± 3.18 abcde</td>
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<tr>
<td>Total No. of cells with MN</td>
<td>4.7 ± 0.33</td>
<td>5.0 ± 0.58</td>
<td>5.3 ± 0.67</td>
<td>80.7 ± 2.97</td>
<td>43.0 ± 3.52 abcde</td>
<td>49.3 ± 3.93 abcde</td>
</tr>
<tr>
<td>Total No. of MN</td>
<td>4.7 ± 0.33</td>
<td>5.0 ± 0.58</td>
<td>5.3 ± 0.67</td>
<td>108.7 ± 4.06</td>
<td>60.6 ± 8.18 abcde</td>
<td>69.7 ± 7.23 abcde</td>
</tr>
</tbody>
</table>

Statistical significance value (P < 0.05).

a, Significant when compared with control group
b, Significant when compared with sennosides 24 mg/ml group
c, Significant when compared with sennosides 48 mg/ml group
d, Significant when compared with radiation group
e, Significant when compared with Rad. & (24 mg/ml) Sennosides group

**Figure 1.** Effect of sennosides (24 & 48 mg/ml) treatment on the total numbers of aberration types induced by γ-rays in human blood cultures.

* Significant when compared with control group
** Significant when compared with radiation group
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Table 2. SOD and CAT activities in human blood cultures (48 & 72 hrs) treated with sennosides (24 & 48mg/ml) after γ-rays exposure.

<table>
<thead>
<tr>
<th>Groups</th>
<th>SOD (U/ml)</th>
<th>Catalase (U/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>48h</td>
<td>72h</td>
</tr>
<tr>
<td>Control group</td>
<td>1.06±0.09</td>
<td>0.9±0.09</td>
</tr>
<tr>
<td>Sennosides (24 mg/ml) group</td>
<td>1.8±0.15a</td>
<td>1.83±0.12a</td>
</tr>
<tr>
<td>Sennosides (48 mg/ml) group</td>
<td>2.17±0.07a</td>
<td>2.27±0.09ab</td>
</tr>
<tr>
<td>Radiation group</td>
<td>0.5±0.15abc</td>
<td>0.47±0.09abc</td>
</tr>
<tr>
<td>Rad. &amp; (24 mg/ ml) Sennosides group</td>
<td>0.93±0.03abc</td>
<td>1.8±0.25abcd</td>
</tr>
<tr>
<td>Rad. &amp;(48 mg/ml) Sennosides group</td>
<td>1.6±0.06abcde</td>
<td>1.67±0.07abcd</td>
</tr>
</tbody>
</table>

Statistical significance value (P < 0.05).

a, Significant when compared with control group
b, Significant when compared with sennosides 24 mg/ml group
c, Significant when compared with sennosides 48 mg/ml group
d, Significant when compared with radiation group
e, Significant when compared with Rad. & (24 mg/ ml) Sennosides group

Figure 2. Cox-2 levels in human blood cultures (48 & 72 hrs) treated with sennosides (24 &48mg/ ml) after γ-rays exposure.
* Statistical significance value (P < 0.05).
** Significant when compared with radiation group

Figure 3. LDH levels in human blood cultures (48 & 72 hrs) treated with sennosides (24 &48mg/ ml) after γ-rays exposure.
* Significant when compared with control group
** Significant when compared with radiation group
DISCUSSION

The world health organization (WHO) has investigated that nearly 80% of the population in the world rely on traditional medicines for meeting their health care needs (17). Deleterious effects of radiation on biological systems have been shown in different ways. Therefore, various strategies have been developed to protect biological systems by means of chemical protection through interfering in the process of radiation damage. Since introduction of cysteine as a radioprotector in 1949, various types of natural and synthetic chemicals were tested for their radioprotective properties on various biological systems by the use of different end points (18).

Calcium sennosides has been widely used in pharmacological research and Folk medicine due to its important medicinal values as laxative...
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& purgative drug (19). Besides, they have been found to exhibit anti-inflammatory, antioxidant (20), hypoglycemic (21), antimalarial, larvicidal (22), antimutagenic (23), and anticancer activities (24).

Direct and indirect action are the main causes of ionizing radiation damages to DNA, where DNA interacts with the reactive free radicals like •OH, •H, and e−aq generated by radiolysis of water in direct action. While indirect action is through production of other free radicals from other molecules. By its role these reactive free radicals can be scavenged by compounds called free radical scavengers thus having the ability to provide protection against damage caused by radiation. From that point of view finding and identifying of natural effective agents which could be used for protection against radiation-induced genetic damage, especially in humans is a very vital target (4).

Leaves and fruits of C. angustifolia contain several laxative constituents such as anthranoids, mostly calcium sennosides A and B, aloe-emodin, emodin, and chrysophanol, fatty oils, flavonoids, polysaccharides and tannins (25). Senna has been widely used as an analgesic, febrifuge, diuretic, hepatoprotective, vermifuge and cholangiole remedy as well as for treating amoebic dysentery, tuberculosis, gonorrhoea, dysmenorrhoea, anaemia, flu, liver and urinary tract diseases (26).

The current study evaluates calcium sennosides antioxidant, antimutagenic, and anti-inflammatory effects on cultured human blood cells. Our results pointed to calcium sennosides treatments with both doses (24 & 48 mg/ml) reduced the numbers of micronuclei, nucleoplastic bridge and nucleoplastic buds) For these reasons, it is now appropriate to refer to this technique as the cytokinesis block MN cytome (CBMN Cyt) assay. So, the presented study used a differential count of mono- and binucleated cells in 1000 to compare the mitotic and the arresting ability of each group with its treatment case. The scored data revealed a low frequency of binucleate cells in radiation group which may be attributed to radiation exposure effects on mitotic and arresting rates.

The present study observations are in agreements with the discussed data that indicated that free radicals arising during radiation-exposure can lead to DNA damage. If overproduction of these radicals occurs, oxidative damage could lead to radiation-induced cytotoxicity; chromosomal damage and micronuclei expression (27). Some phytochemical and herbal drugs may influence this process by controlling the formation of the damaging agents in certain circumstances, while other components, such as hormones may function to enhance repair mechanism (28).

The current study also displays the effects of γ-irradiation on human blood cell cultures (48 & 72h) which revealed significant inhibition in SOD & CAT activities compared with control group due to heavy oxidative burden induced by oxidative stress as result of free radical production. While, groups treated with calcium sennosides after irradiation recorded significant improvements in mentioned parameters values as compared with irradiated group (table 2). That observed enhancement of SOD activity recorded after calcium sinrisitis treatment goes hand to hand with the results found by Shanmugasundaram (5).

Imbalance between pro-oxidant and antioxidant status in the cells following to ionizing radiation was defined by Bhosle (29). In addition to decrease in SOD activities as a results of attacking its molecules with produced Reactive Oxygen Species (ROS) causing their denaturation and partial inactivation (30). Also, the radioprotective effect and the antioxidative potentials of calcium sennosides have been demonstrated in several study’s findings as a reduction in MN, and improvement of SOD and CAT levels (6). Moreover, the inflammatory component of most human inflammatory chronic
The medicinal action of senna can be attributed mainly to the anthraquinone glycosides, especially calcium sennosides. It appears that the aglycone portion is responsible for its action. The breakdown of the anthraquinone glycosides in the digestive tract can occur in one of two ways. The bowel flora can directly hydrolyze them in a similar way to that of free active aglycone. Alternatively, in the presence of bile and the sugar moiety, the free aglycone can be absorbed into the blood stream and secreted later into the colon (2).

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