Productivity, Stability and Anti-Recombinagenic Activity of Cassia fistula Pods Pulp Extract as a Source of Natural Food Color

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Abstract: This study aims to take advantage of the aqueous extract of Cassia fistula pods pulp, which is used in folk medicine, in order to obtain a higher extractive value under different conditions of temperatures and pHs. The extract obtained from the significant highest extraction trials was subjected to test its stability under different conditions of temperatures and pHs. Thermal stability and brown substance determination on extracts were also studied. The mutagenic, recombinagenic, carcinogenic and anticarcinogenic potential of Cassia fistula pods extract was assessed using the epithelial tumor detection test (wts) in D. melanogaster. Results of the study showed that the highest extractive value was 67.47% at 90°C, pH 7 for 20 min. The extract exhibited high stability at different temperatures and pHs and in particular on the neutral and alkaline pH as well as high temperatures. Generally, the extract showed a significant high time and thermal stability at 80 and 90°C with the increase of extraction time at pH 7 or 9. The significant highest brown substance units after extraction for 30 min were at 90, 80 and 60°C. Studies on the effect of extracts on the genetic toxicology showed that descendants treated with extracts did not show any statistically significant changes in the frequency of tumors when compared to the negative control. Therefore, the results indicate that the C. fistula extracts, under the present experimental conditions, did not induce the occurrence of tumors in D. melanogaster.

Key words: Cassia fistula • Pods • Natural color • Antitumor activity

INTRODUCTION

Natural colors have always been part of the diet and it is receiving growing interest from both food manufactures and consumers in continuing replacement of synthetic colors. Chlorophylls, carotenoids and anthocyanins are consumed in the foods. Common natural colorings include annatto, saffron, paprika, grape skins, caramel, beetroot, cochineal and turmeric. Natural colors add a cachet to food products marketed as “natural” and “organic” that suit the shoppers demand and growing health-conscious of populations. Nevertheless, natural colors are perceived by the consumer as being less of a health hazard than the petroleum- (coal-tar) derived synthetics, with names like tartrazine, indigotine and erythrosine. The use of non-permitted colors is known to cause adverse effects in experimental animals [1, 2, 3] and in humans [4, 5]. Repeated exposure to even the permitted synthetic colors may be hazardous [6]. Cassia fistula Linn. is fast-growing, medium-sized, deciduous tree belongs to Fabaceae family and commonly known as Indian Laburnum, golden shower in English and Amultas in Hindi [7]. It is distributed in various regions including Asia, South Africa, China, West Indies and Brazil as well as in Egypt [8, 9]. The fruit is cylindrical pod containing many horizontal black seeds. The ripe pods are pendulous, cylindric, smooth, shining and of brown-black color [10]. C. fistula could be one of the alternative or complementary therapies particularly because of its low toxicity and its widespread use for its multiple medicinal effects. The pulp from pods is of great therapeutic value, it is a mild, pleasant and safe purgative due to presence of sennoside and rhein [11, 12], even for children and expectant mothers. Crude hydro-alcoholic extract of Cassia fistula fruit pulp has high antioxidant activity and antifungal action as a result of its high phenolic and flavonoid content [13-16].

Pulp of cassia fruits has high concentrations of soluble sugars, volatile oils, waxes, resinous substances and it contains several anthraquinones such as rhein,
aloin, emodin and sennosides [17, 18]. Fistulic acid (an anthraquinone acid) was detected in *Cassia fistula* pods which are characterized as a structure of a new coloring matter [19].

The evaluation of toxic properties of *C. fistula* is crucial when considering public health protection because exposure to plant extracts can result in undesirable effects on consumers [20]. Various assays are used in field of genetic toxicology to assess the damage on the DNA, in the presence or absence of mass metabolism systems. These assays include the epithelial tumor clones test (wts) in *Drosophila melanogaster* developed by Eeken et al. [21]. The use of wts test can detect the carcinogenic of chemical or natural and synthetic agents that induced by genetic abnormalities such as mutation, deletion and recombination. This assay is based on warts tumor suppressor gene that encodes protein kinase depends on serine and threonine. Deletion or loss of wts gene leads to the formation of epithelial tumors that appears as homozygotic clones in heterozygotic flies which develop throughout the fly body cuticles [21-24]. The system identifies and characterizes the potential tumorigenesis compounds and scores for loss of heterozygosity (LOH). LOH occur due to different types of recombination, deletions, point mutations and loss of chromosomes or nondisjunction [22]. Heterozygous loss in the cells of the drosophila imaginable disks is associated with homozygous clone formation in the larvae, leading to visible phenotypes in the eyes and wings in the adult flies. Because consumers seek to take natural therapeutic substances and reduce synthetic materials in their food, so this work is a trial aims to obtain the highest crude pulp extract yield of *Cassia fistula* fruits with the optimum temperature and pH conditions. The effect of temperature and pH on selected crude extract stability was also evaluated. Also, Crude extract is evaluated as a source of brown color substance. Genetic toxicology of crude extract is determined to assess potential alterations in DNA associated with its using in vivo epithelial tumor detection test (wts) on *Drosophila melanogaster*.

**MATERIALS AND METHODS**

Pods of *Cassia fistula* were collected from the campus of Faculty of Agriculture, Ain Shams University, during June 2014. Caramel used in this study was purchased from Aarkay Food Products Ltd. Company, India. *Drosophila melanogaster* strains were provided by Bloomington Drosophila Stock Center of the University of Indiana, USA, under registry No. (Bloomington/7052). Two *D. melanogaster* strains were used for the experiments: (1) Oregon R: OR, wild type strain and (2) wts/TM3, sb1 strain. Due to lethalness of the warts (wts) allele, wts<sup>4021</sup> allele on chromosome 3 is balanced over TM3 chromosome, with multiple inversions and marked by the dominant stubble (Sb) mutation, phenotypically identified by short bristles according to Eeken *et al.* [21]. The genetic structure of this strain is; st p in ri wtsMT4-1/TM3 Sb, which was abbreviated as wts/TM3. The genetic symbols about the various markers and the balancer chromosome can be found in web site (flybase.bio.indiana.edu) [25, 26].

**Preparation of *C. fistula* Pods Pulp Extract:** The pods pulp was separated and its moisture content was determined according to Reshmi *et al.* [28]. A 10g fresh pods pulp was extracted in 100 ml buffer solution at different pHs (3, 5, 7 and 9) and temperatures (10, room temperature (RT, 30°C±3), 60, 80 and 90°C) for 20 min and in addition, samples which extracted at 10°C and RT were allowed to stand overnight. The extracted samples were centrifuged at 4000 rpm for 5 min to remove suspended particles then dried under vacuum. The extractive value was calculated according to the following equation:

\[
\text{Extractive value (\%) = \frac{\text{dry weight of extract}}{\text{dry weight of fresh pulp}} \times 100}
\]

A spectrophotometric scan was carried out in the range of 320 to 720 nm for the crude extract and the maximum absorbance was observed at 560 nm.

**Crude Extracts Stability at Different Temperatures and pHs:** For the stability studies of *Cassia fistula* pods pulp crude extract, the aforementioned extraction method applied at 90° C and pH 7 for 20 min were used to obtain the necessary extract for this study. The vacuum dried extract was dissolved in different pH buffers (3, 5, 7 and 9) at ratio 1:10 then incubated under different temperatures, 30 ±3 (RT), 40, 60, 80 and 90°C, for 30 min according to Reshmi *et al.* [28]. Samples were cooled and centrifuged at 4000 rpm for 5 min then the supernatant of each extract was volumetrically doubled with its buffer solution. The absorbance was measured at 560 nm and caramel color solutions (at the same conditions) were used as controls.

**Time and Thermal Stability of Crude Extracts:** The aforementioned crude extract was dissolved in buffer solutions (pH 7 and 9) with 1:10 ratio and held at 80 or 90°C for 180 min [29]. The samples were withdrawn at 30 min
intervals then cooled immediately in an ice bath followed by centrifugation at 4000 rpm for 5 min. The supernatant of each extract was volumetrically doubled with its buffer solution and the absorbance was measured against caramel as a control at 560 nm.

**Determination of Brown Substances:** Brown substances were determined in aqueous crude extracts of cassia pods pulp (10 mg/ml) at pH 7 with different extraction temperatures (30, 60, 80 and 90°C) for 10, 20 and 30 min according to Alberto [30]. The obtained extracts were centrifuged as mentioned before and the absorbance was measured spectrophotometrically at 365 and 456 nm and the concentration of brown substances was calculated by the following equation:

Brown substances (units) = 1000 (6.50 A<sub>365</sub> – A<sub>456</sub>) /6.36 C

where:
C = concentration of the sample in mg/ml.

**Detection of Epithelial Tumor in D. melanogaster:** This assay was conducted to identify and characterize the potential tumorigenesis of *C. fistula* pods crude extract and scores for loss of heterozygosity (LOH) on *D. melanogaster*.

**Cross and Experimental Procedure of Larval Treatment:** Heterozygous larvae with genetic structure wts/+ were obtained by crossing virgin females wts/TM3, Sb1 with wild type males +/+. Eggs obtained from of the cross were collected during a 6 h period. After 72± 4h, Heterozygous larvae were cleaned from remaining feeding medium with a 20% glycerol solution and they were transferred to treatment vials containing Drosophila medium supplemented with 500 mg of *C. fistula* pods pulp extracts which were previously extracted at different temperatures and pHs. The extracts were dissolved in 2 ml of distilled water then mixed well in 98 ml of standard Drosophila medium at 50°C (5 mg/ml) for 24 h, then they were transferred to standard Drosophila medium [21, 23]. Doxorubicin, DXR (0.125 mg/ml) was used for the positive control and water for the negative control. Only adult flies, without the chromosome balancer (TM3, Sb1) were analyzed. All Drosophila stocks and crosses were maintained at 25°C ±2°C and 65% humidity.

**Scoring of Tumors:** Adult males and females flies of the (wts/+ ) genotype, which had wild hairs (long and thin) were analyzed for tumor (wart) presence. The flies were observed using a Leica stereoscopic at a standard magnification of 25X. Only tumors that were large enough to be unequivocally classified were recorded. The tumor frequency was calculated as the number of tumors/number of wts/+ flies [21, 31].

**Statistical Analysis:** Each extraction and stability trial was carried out in triplicate. The experimental data were subjected to an analysis of variance for a completely random design using a Statistical Analysis System [32]. Duncan’s multiple range tests were used to determine the difference among means at the level of 0.05.

**RESULTS AND DISCUSSION**

**Extractive Value:** *Cassia fistula* pods pulp was extracted by aqueous buffers with different temperatures and pHs. The yield percentages are shown in Table 1. From the point of temperature effect under the pH, an increase in extraction temperature increases the efficiency of the extraction yield. Since heat render the cell wall permeability, increase solubility and diffusion coefficients of the compounds to be extracted. The highest extractive values were 38.42, 43.14, 67.47 and 56.15% at 90°C for pH 3, 5, 7 and 9, respectively. According to the effect of pH on extractive value under the same temperature, the data showed that with the increase of extraction buffer alkalinity, the extractive value was increased except at

<table>
<thead>
<tr>
<th>pH</th>
<th>10%</th>
<th>10/O</th>
<th>RT</th>
<th>RT/O</th>
<th>60%</th>
<th>80%</th>
<th>90%</th>
<th>Rate of increase (% ^°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>11.23&lt;sup&gt;a&lt;/sup&gt;</td>
<td>29.55&lt;sup&gt;b&lt;/sup&gt;</td>
<td>31.91&lt;sup&gt;b&lt;/sup&gt;</td>
<td>33.69&lt;sup&gt;c&lt;/sup&gt;</td>
<td>35.46&lt;sup&gt;cd&lt;/sup&gt;</td>
<td>36.64&lt;sup&gt;cd&lt;/sup&gt;</td>
<td>38.42&lt;sup&gt;cd&lt;/sup&gt;</td>
<td>2.519x10^-3</td>
</tr>
<tr>
<td>5</td>
<td>26.00&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>28.96&lt;sup&gt;cd&lt;/sup&gt;</td>
<td>30.15&lt;sup&gt;cd&lt;/sup&gt;</td>
<td>35.46&lt;sup&gt;b&lt;/sup&gt;</td>
<td>36.05&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>39.60&lt;sup&gt;cd&lt;/sup&gt;</td>
<td>43.14&lt;sup&gt;cd&lt;/sup&gt;</td>
<td>3.883x10^-3</td>
</tr>
<tr>
<td>7</td>
<td>38.42&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>41.37&lt;sup&gt;cd&lt;/sup&gt;</td>
<td>38.42&lt;sup&gt;cd&lt;/sup&gt;</td>
<td>39.01&lt;sup&gt;b&lt;/sup&gt;</td>
<td>41.96&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>43.74&lt;sup&gt;cd&lt;/sup&gt;</td>
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<td>1.187x10^-2</td>
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<tr>
<td>9</td>
<td>41.37&lt;sup&gt;cd&lt;/sup&gt;</td>
<td>39.60&lt;sup&gt;cd&lt;/sup&gt;</td>
<td>35.46&lt;sup&gt;b&lt;/sup&gt;</td>
<td>39.01&lt;sup&gt;b&lt;/sup&gt;</td>
<td>43.14&lt;sup&gt;cd&lt;/sup&gt;</td>
<td>46.69&lt;sup&gt;cd&lt;/sup&gt;</td>
<td>56.15&lt;sup&gt;cd&lt;/sup&gt;</td>
<td>6.585x10^-3</td>
</tr>
</tbody>
</table>

Means with the same capital letter in the same column (pH) or the same small letter in the same row (temperature) are significantly different at (p = 0.05). RT: room temperature, 30±3°C; O: overnight.
Time and Thermal Stability of Crude Extracts: Time and thermal stability of aqueous crude extracts of *Cassia fistula* pods pulp as affected by two temperatures (80 and 90°C) and pH-values (7 and 9) over a period of 180 min was presented in Fig. 2. As seen, the absorbance of control samples (caramel) tested at pH 7 or 9 was almost stable at 80 and 90°C for 180 min. On other side, the crude extracts of *C. fistula* pods pulp showed significant lower absorbance level compared with their control samples at the tested pH, temperature and time periods. The absorbance of crude extracts at 90°C being higher than those measured at 80°C at pH 7 till 120 min. However, a non-significant drop in the absorbance of crude extract (8.24%) was observed after 120 min of heating at 90°C and
Fig. 3: Brown substances (units) of *Cassia fistula* pods pulp at different temperatures and extraction times. Means with the same letter are significantly different at (p = 0.05).

<table>
<thead>
<tr>
<th>Treatments</th>
<th>No. of F1 files scored</th>
<th>Number of wts tumors scored</th>
<th>Frequency (No. of wts tumors/fly)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative control</td>
<td>2376</td>
<td>60</td>
<td>0.025</td>
</tr>
<tr>
<td>DXR (0.125 mg/ml)</td>
<td>450</td>
<td>410</td>
<td>0.911***</td>
</tr>
<tr>
<td>pH 5</td>
<td>80°C</td>
<td>1001</td>
<td>32</td>
</tr>
<tr>
<td></td>
<td>90°C</td>
<td>963</td>
<td>35</td>
</tr>
<tr>
<td>pH 7</td>
<td>10°C</td>
<td>1325</td>
<td>37</td>
</tr>
<tr>
<td></td>
<td>RT</td>
<td>1557</td>
<td>35</td>
</tr>
<tr>
<td></td>
<td>90°C</td>
<td>1150</td>
<td>20</td>
</tr>
<tr>
<td>pH 9</td>
<td>RT</td>
<td>1594</td>
<td>38</td>
</tr>
<tr>
<td></td>
<td>80°C</td>
<td>1981</td>
<td>56</td>
</tr>
<tr>
<td></td>
<td>90°C</td>
<td>1141</td>
<td>31</td>
</tr>
</tbody>
</table>

*The value is considerably different from the negative control (P<0.05).

P**

Brown Substances of Crude Extracts: Brown substance units of *Cassia fistula* pods pulp extracts at pH 7 with different temperature (30, 60, 80 and 90°C) for 10, 20 and 30 min extraction time were determined and the results are presented in Fig. 3. As seen, brown substance units were significantly increased from 135, 145, 146.6 and 153 for 10 min extracts obtained at 30, 60 and 80°C, respectively to 154, 158, 159.4 and 159 units for the corresponding extracts obtained after 30 min of heating. After 10 min of extraction time, the significant highest brown substance units were 153 for extract obtained at 90°C. There was no significant difference in brown substance units after 30 min at 60, 80 and 90°C. These data were in agreement with those of Nie et al. [35].

**Genetic Toxicology of Cassia fistula Pods Pulp Extract:**
The potential tumorgenesis of *C. fistula* aqueous extracts were tested using *in vivo* epithelial tumor detection test (wts) on *Drosophila melanogaster*. The wts-epithelial tumor detection test results for the chronic treatment of larvae treated with 5mg/ml *C. fistula* aqueous extract are shown in Table 2. Warts tumors were induced in wts/+ adult flies in frequencies of 0.911 per fly by doxorubicin (DXR 0.125 mg/ml) as positive control, extremely statistically significant above the control according to x2 -test. In the 410 flies analyzed and treated with DXR, 450 tumors were identified. These tumors arose in every part of the fly analyzed (eyes, head, wings legs and halters). Meanwhile, the frequency of tumors in the wts/+ control flies was low (0.02–0.03; i.e. 2–3 flies with one wart in 100 flies scored). In the negative control experiment, the
frequency of spontaneous tumors was low, where 60 small tumors were scored among 2376 flies with an average of 0.025 tumor /fly, indicating a spontaneous frequency. Descendants treated with different*C. fistula* aqueous extracts obtained under different levels of pH and temperatures, did not show any significant changes in the frequency of tumors, when compared to the corresponding negative control. Therefore, no significant carcinogenic effect of *C. fistula* aqueous extract was found. On the other hand, DXR (used as a positive control, enhanced the frequency of tumors (*P*<0.05). Carcinogenicity for extracts were carried out to confirm quality criteria for food quality and safety and their potential utilization in multicomponent biological/food systems as well as their possible use in the clinical treatment of several human malignancies. Induction of tumors using wts- epithelial tumor detection test allows one to draw closer analogy between the activity of a substance in Drosophila and its potential carcinogenic hazard to humans [22]. Gupta et al. [36] reported the antitumor activity of methanolic extract (ME) of *cassia fistula* seeds on the growth of Ehrich Ascites Carcinoma (EAC) and on the life span of tumor bearing mice. ME treatment showed an increase of life span and a decrease in the tumor volume and viable tumor cell count in the EAC tumor host. The same results were obtained by Bahorun et al. [17], Jothy et al. [20], Duraiyandiyana et al. [37] and Luximon et al. [38]. Anti-tumour activity of *C. fistula* seed extract based on cytological studies reveal that a reduction in the mitotic activity can be the leading mechanism of action against tumorigenesis. Indeed the appearance of membrane blebbing and intracytoplasmic vacuoles in the treated tumour cells suggest that these pathways may account for the reduction in tumor volume [36, 39].

From the previous data it can be deduced that the high therapeutic crude extract of *C. fistula* pods pulp may be used as a source of natural brown color with antitumor activity.

REFERENCES


25. FlyBase, C., 2008. Insertion Identifiers and Alleles Based on Genomic Location of Insertions With Respect to Gene Annotations. (http://flybase.org/).


