Evaluation of Genotoxicity of *Cassia occidentalis* in Rodents

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ABSTRACT:

Genotoxic potential of *Cassia Occidentalis* (CO-A002) leave extract was studied by in vivo assays system. In the present study, genotoxicity was evaluated in rat using different cytogenetic assays according to OECD guideline number 474, 475 and 489 i.e., (a) Micronucleus assay (b) Chromosomal aberrations (c) Comet assay (d) Sperm abnormalities assay respectively. Cyclophosphamide (40mg), mercuric chloride (0.864mg) and metronidazole (125mg) /kg p. o. were used as standard drugs as per the experiments, whereas CO-A002 was given at 100, 200 and 400 mg/kg respectively. Results reveals that CO-A002 did not produce any adverse effect and is safe at a dose of 400 mg/kg p.o.

Keyword: Cassia Occidentalis, Chromosomal aberrations, Micronuclei, Comet, Sperm abnormality.

INTRODUCTION

Nowadays, toxicity and safety of medicinal herbs have become one of the most discussed topics all over the world. As per the guideline of Committee on Herbal Medicinal Products on Traditional herbal medicine (a safety profile in accordance to modern standards is supported by their documented history of medicinal use). Globally natural products played pivotal role for the treatment and prevention of number of diseases. Most of the modern therapeutic drugs have herbal origin. However, limited safety profile information lead to side effects and even death. There is a misconception that medicinal plants being natural are commonly safe and free from undesirable side effects but the scientific data reported that the natural products which are being used on large scale for the treatments of specific diseases are known to have side effects also. However, the potential toxicity of plant has not been recognized by the general public or by professional groups of traditional medicines. [Xie, 2011 #1; Farnsworth, 1988 #2; Assessment, 2003 #3] This ignorance leads to development of idiosyncratic adverse events due to consumption of such plants. Thus, evaluating the toxicological effects of any phyto-extract intended to be used in humans is of utmost importance and requirement for the regulatory purposes. Furthermore, it is observed that toxicity and safety profile of plant based medicines is one of the most discussed topics as herbal products have become popular globally. Approximately 80% of world population till date depends on herbal medicines for the treatment of common ailments. *Cassia Occidentalis* (CO) commonly called as kasondi or kasamarda or bardi haedma is one of such herbs and is used globally. It possesses \(^1\) Antidiabetic, anti-inflammatory \(^2\)hepatoprotective, \(^3\)anti larvicidal, \(^4\)Antiplasmodial, \(^5\)antihelmintic, \(^6\)analgesic and \(^7,8\)antifungal (ringworm) activities. \(^8\) The leaves paste is also applied for fracture healing and also on injure site for relief.

The hereditary material of the organism is the DNA which remains under stress continuously in the environment. The stressful conditions of the environment have the ability to damage the DNA. The living organisms possess DNA repairing mechanisms in their cells fortunately. The individuals suffer from different disorders associated with DNA abnormalities, if there is no repairing mechanism present in the
cells. A failure of DNA repair mechanisms is accountable to either illnesses or any sort of cancer. Researchers believe that there is a casual link between failure of DNA repair mechanism of organism and onset of a cancer. Many questions remain unanswered, general principle of the sequence of events which leads to cancer after exposure to genotoxic carcinogens has become increasingly obvious.

CO-A002 contains flavones, quercitin, quercitrin, campferol, rutin, caffeic acid, ferulic acid, coumaric acid and gallic acid etc which are responsible for its therapeutic potential.

The main objective of the study is to evaluate the genotoxicity of the leaves extract of CO-A002 i.e., anti-mutagenic potential because the extract of CO-A002 is consumed by different age people without known its deleterious effects. Keeping above therapeutic potential and usage of CO-A002 into consideration, the study was designed to investigate the genotoxic potential of CO-A002.

MATERIAL AND METHOD:

Chemical & Reagents:
Phosphate buffer saline (PBS; Free from calcium and magnesium), cyclophosphamide, ethidium bromide, Giemsa stain, Eosin-Y, Trizma base, Acridine orange, EDTA, mercuric chloride, low melting point agarose and routine agarose were purchased from Sigma Aldrich (electrophoresis grade). Metronidazole, cyclophosphamide were obtained from Nicolas Piramal India Ltd.

Extract Preparation:
Leaves of Cassia occidentalis were collected from Akhnoor area of Jammu in the month of August and September, shade dried and crushed into fine powdered. The powdered leaves (quantity in gms) were extracted with 500ml 50% Ethanol. The extract was filtered and filtrate was freeze dried using freeze dryer into amorphous powder.

Animals
Male Wistar rats (140-160g) were used and kept at 24±2°C under 12:12 hr light and dark cycles with free access to pellets food and water in the animal house of Indian Institute of Integrative Medicine. All experiments were conducted according to ethical guidelines approved by Institutional animal ethics committee.

Leaves Were Oven-dried

Finely Powdered (100 gm)

Extracted With 500 ml 50% Ethanol

Filtered & Filtrate collected

Evaporated Using A Rotary Evaporator

Dried Using A Freeze-dryer

Fig 1: Extraction Protocol of Cassia Occidentalis
Bone marrow chromosome assay
Five groups of animals, each comprised of five rats were taken for the study of chromosomes aberration. Group-1 received normal saline, group -2 received Cyclophosphamide (CP) 40mg/kg p.o. as positive control whereas groups 3, 4 and 5 received 100, 200 and 400mg/kg p.o. of CO-A002 respectively, prepared as homogenized suspension in 2% gum acacia. Animals were treated for 15-days whereas control group received 2% gum acacia as a vehicle in a fixed volume of 1ml/100g. All animals were given colchicines intraperitoneal (4mg/kg), 24h after last dose, to arrest the cell division. After 2 h of colchicines injection, animals were sacrificed. Metaphase cells preparations were obtain from bone marrow cells with slight modifications. Briefly, bone marrow was aspirated or flushed out from femur bone with 1ml of 0.075M KCl. The bone marrow suspension was incubated at 37°C for 20 minutes and centrifuged at 65 g for 10 minutes. The pellet obtained after discarded suprenant was mixed with freshly prepared Carnoys fixative (3:1, solution of methanol and acetic acid) and allowed to stand for 15 min. followed by centrifugation. The preparation was given two washings with fixative (500µl in each washing). The resultant pellets were mixed 500 µl fixative and 20 ul of suspension was dropped from distance of 25 cm in the glass slide to facilitate the bursting of the cells. These slides were allowed to dry and stained with 10% Giemsa stain at pH 6.8 for 5 min. and screened for any abnormalities of chromosomes.

Micronucleus assay in bone marrow
Treatment schedule of CO-A002 extract administration remained the same as that of bone marrow chromosome assay. The group-1 was given gum acacia (1ml/100g) and group-2 was given Cyclophosphamide (40mg/kg) as positive control, whereas groups 3, 4 and 5 were administered extract of CO-A002 (100,200 and 400mg/kg) respectively p.o. for fifteen days. The animals were sacrificed and bone marrow was aspirated in 1ml FBS (fetal-bovine-serum). After centrifugation at 65 g, the cells were re-suspended in 100µl FBS. Smear was prepared and fixed with methanol. The slides were stained with acridine orange. Around 2000 cells were evaluated to determine the numbers of micronucleated polychromatamatic erythrocytes (MNPE) under fluorescent microscope (Olympus IX75, Japan). In order to access the possible toxic effects of the treatments on erythropoisis %age of polychromatic erythrocytes (PEC) were evaluated over 1000 total erythrocytes (PCE+NCE) (NCE= Normochromatic Erythrocytes).

Comet assay
Effect of test compound on DNA fragmentation was investigated by the method described by Singh et al.1988 with slight modification. The treatment schedule of CO-A002 extract administration remained the same as that of bone marrow chromosome assay except for the change in the positive control which in this case was HgCl₂ (0.864mg). 50µl of EDTA blood from the control and treatment groups was embedded in 450µl of low melting point (LMP) agarose (0.75g/100ml) and resulting mixture was spread over a pre coated microscopic plane slides (0.1% Agarose routine).The gel was covered with a glass cover slip and kept to set at 4 C° for 5-10min. Gel embedded cells were lysed in lysing solution (2.5MNaCl, 100mM disodium EDTA, 10mM trizma base, 8g/l NaOH, PH adjusted to 10 by using NaOH or HCl) for 20 min at 4 C°, to allow DNA unwinding. Electrophoresis was performed at 100mA and 30 V for 20 min. The slides were stained with Ethidium bromide (EtBr 20µg/ml) and observed under fluorescence microscope 1x 40 equipped with an excitation filter (BP 510 nm) and a barrier filter (590nm). Analysis was done using KOMET software (Version 5.5). Tail moment values obtained were used in order to evaluate the amount of DNA damage/fragmentation. At least, one hundred cells were scored for each individual. Generally, three parameters were used to access the damage of DNA such as (1) olive tail moment (2) tail length and (3) tail coefficient variance.

Sperm abnormality assay
Five groups of animals each comprised of five rats were taken for the study. First control group received gum acacia, group 2 received metronidazole (MTZ) 125mg/kg as positive control whereas 3,4 and 5 received CO-A002 leaves extract at doses (100, 200 and 400mg/kg) respectively by oral gavage.
hr of last dose administration, the animals were sacrificed and epidedemis excised by laprocomy and sperms suspension was formed in 1ml saline by mincing the epidedemis. The suspension was filter through 80 µm nylon mesh for preparing smears for the evaluation of sperm shape abnormalities after staining with Eosin-Y(0.1%) for 30 minutes. The morphological abnormalities of the sperm head shape and other changes were enumerated in accordance with Wyrobek and Bruce method (1975) by applying binocular light microscope at 100x magnification. 500 sperms were counted for morphological changes in each animal. Dat obtained was represented as percent total sperm morphological abnormality.

**Statistical analysis**

The data obtained were calculated and Statistical analysis was done using One Wat ANOVA followed by student-Newman-Keuls test for multiple comparison using Prism software (Version 5.0). Values are considered to be significantly different at p<0.05.

**RESULTS**

**Bone marrow chromosome assay**

Data was analysed at the end of treatment on day fifteen. control group showed a few aberrant metaphases (inclusive of metaphase with gaps), whereas positive control group showed statistically significant aberrant metaphases and chromosome aberrations The incidence of chromosome aberrations in CO-A002 treated groups were mostly fragments, sister chromatids exchanges and ring formation by all three treatment doses but found statistically insignificant when compared to vehicle control group (Table 1, Figure 2).

**Figure 2.** Metaphase plates of different treated groups.(A) normal group (B) cyclo-40mg treated (C) CO treated group

**Table 1:** Chromosomal abnormalities in bone marrow cells of rats treated with 3 doses of CO-A002 and positive control CP-40mg/kg.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Control</th>
<th>Cyclo-40mg/kg</th>
<th>CO-A002-100mg/kg</th>
<th>CO-A002-200mg/kg</th>
<th>CO-A002-400mg/kg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>86.01±1.6</td>
<td>43.34±2.6</td>
<td>87.34±2.6</td>
<td>88.05±0.8</td>
<td>84.17±2.4</td>
</tr>
<tr>
<td>Fragment</td>
<td>6.3±0.33</td>
<td>16.67±2.6</td>
<td>4.33±0.33</td>
<td>4.84±1.20</td>
<td>7.15±1.30</td>
</tr>
<tr>
<td>GAP</td>
<td>1.91±0.09</td>
<td>7.34±0.88</td>
<td>2.34±0.88</td>
<td>1.48±0.03</td>
<td>1.98±0.09</td>
</tr>
<tr>
<td>Sister chromatids exchange</td>
<td>5.48±0.45</td>
<td>22.53±0.74</td>
<td>5.54±1.03</td>
<td>4.82±0.95</td>
<td>6.34±1.20</td>
</tr>
<tr>
<td>Breaks</td>
<td>-------</td>
<td>4.0±0.70</td>
<td>-------</td>
<td>----</td>
<td>----</td>
</tr>
<tr>
<td>Rings</td>
<td>0.3±0.03</td>
<td>2.77±0.9</td>
<td>0.45±0.0</td>
<td>0.81±0.4</td>
<td>0.90±0.5</td>
</tr>
<tr>
<td>Others</td>
<td>-------</td>
<td>2.77±0.9</td>
<td>-------</td>
<td>----</td>
<td>----</td>
</tr>
<tr>
<td>Total</td>
<td>13.99±1.57</td>
<td>56.66±3.36</td>
<td>12.66±1.13*</td>
<td>11.95±2.46*</td>
<td>16.43±1.66*</td>
</tr>
</tbody>
</table>

Values are the means ±SE (N=5).* means P<0.05 when compared with positive control using one way ANOVA followed by Student-Newman-Keuls Test for multiple comparison.
Bone marrow micronucleus assay

CO-A002 did not show any significant change in the percentage of PCEs, MNPCEs or MNNCEs at any dose levels (100-400mg/kg p.o.) in comparison to the vehicle control, whereas CP (40 mg/kg p.o.) showed a significant decrease in the percentage of PCEs whereas percentage of MNPCEs and MNNCEs was found to be increased (Table 2, Figure 3 & 4).

<table>
<thead>
<tr>
<th>Treatment Table</th>
<th>PCEs</th>
<th>MNPCEs</th>
<th>MNNCEs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>19.5±1.32</td>
<td>4.25±0.75</td>
<td>4.75±0.63</td>
</tr>
<tr>
<td>CP-40mg/kg p.o.</td>
<td>13.25±2.8</td>
<td>18.5±2.21</td>
<td>10.0±1.68</td>
</tr>
<tr>
<td>CO-A002-100 mg/kg p.o.</td>
<td>20.0±1.29</td>
<td>3.5±0.64</td>
<td>4.0±0.70</td>
</tr>
<tr>
<td>CO-A002-200 mg/kg p.o.</td>
<td>23.25±1.54</td>
<td>4.25±1.10</td>
<td>3.25±0.85</td>
</tr>
<tr>
<td>CO-A002-400 mg/kg p.o.</td>
<td>23.75±1.54</td>
<td>4.5±0.64</td>
<td>5.0±1.08</td>
</tr>
</tbody>
</table>

Values are the means ±SE (N=5).* means P<0.05 when compared with positive control using one way ANOVA followed by Student-Newman-Keuls Test for multiple comparison.

Figure 3: Showing MN with different treatments in bone marrow of rat.

Figure 4: Graphical representation of percentage of micronuclei frequencies or number (Polychromatic Erythrocytes, Micronucleated Polychromatic Erythrocytes and Micronucleated Normochromatic Erythrocytes) in different treated groups.

Table 2: Comparison of percentage of micronuclei frequencies or number (Polychromatic Erythrocytes, Micronucleated Polychromatic Erythrocytes and Micronucleated Normochromatic Erythrocytes) in different treated groups.
Comet assay:
The results of comet assay are given in the table 3. The data obtained did not show any significant changes in olive tail moment (OTM) values at any dose of CO-A002 in peripheral blood of the treated rat. The OTM value at a dose of 100, 200 and 400mg/kg p.o. of CO-A002 extract was 0.61, 0.55 and 0.53 respectively in comparison to the control group value 0.44. On the contrary positive control treated with HgCl$_2$ (0.864mg) exhibited highly significant increase in the OTM values that was more than 10 folds of the vehicle control (Table 3).

**Table 3: DNA damage analysis by Comet assay in peripheral blood of rats.**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Head DNA(%)</th>
<th>Tail DNA(%)</th>
<th>OTM</th>
<th>Tail Length(µm)</th>
<th>Tail Coefficient</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>94.68±0.54</td>
<td>6.25±0.77</td>
<td>0.31±0.01</td>
<td>1.21±0.02</td>
<td>1.23±0.02</td>
</tr>
<tr>
<td>HgCl$_2$ (0.864mg/kg)</td>
<td>81.72±1.68</td>
<td>19.25±0.9</td>
<td>1.17±0.58</td>
<td>14.12±1.53</td>
<td>3.85±0.66</td>
</tr>
<tr>
<td>CO-A002-100 mg/kg p.o.</td>
<td>95.22±0.69</td>
<td>3.95±0.42</td>
<td>0.35±0.01</td>
<td>1.15±0.01</td>
<td>1.72±0.02</td>
</tr>
<tr>
<td>CO-A002-200mg/kg p.o.</td>
<td>94.92±1.25</td>
<td>5.52±0.34</td>
<td>0.42±0.01</td>
<td>1.16±0.02</td>
<td>1.90±0.03</td>
</tr>
<tr>
<td>CO-A002-400mg/kg p.o.</td>
<td>94.0±0.57</td>
<td>5.92±0.52</td>
<td>0.45±0.02</td>
<td>1.24±0.11</td>
<td>1.89±0.02</td>
</tr>
</tbody>
</table>

OTM - Olive tail Moment. Values are the means ±SE (N=5).* means P<0.05 when compared with positive control using one way ANOVA followed by Student-Newman-Keuls Test for multiple comparison

**Sperm abnormality Assay**
The results of the sperm abnormality are shown in the table. The abnormalities which were take into consideration are folded or coiled filament, amorphous, flagellum with ansa, double head, double tail, coil with microcephaly, bent at cephalocaudal region, hookless flagella and multiple abnormalities (Figure 5). No significant change in any of the observed abnormalities was recorded at any dose level of extract of CO-A002 treated groups. The positive control (MTZ) on the contrary exhibit significant change in all the observed abnormalities (Table 4, Figure 6).

![Figure 5: Morphological abnormalities observed in spermatozoa of mouse.](image)

(A) normal sperms (B) cephalocaudal bend (C) duplication of tail (D) duplication of head.
Table 4: Sperms analysis for sperm abnormality test.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Control</th>
<th>MTZ-125 mg/kg p.o.</th>
<th>CO-A002-100 mg/kg p.o.</th>
<th>CO-A002-200mg/kg p.o.</th>
<th>CO-A002-400mg/kg p.o.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>81.08±2.27</td>
<td>43.75±1.75</td>
<td>85.25±1.70</td>
<td>86.5±2.70</td>
<td>87.5±2.98</td>
</tr>
<tr>
<td>Coiled/Folded</td>
<td>9.5±0.64</td>
<td>6.25±1.10</td>
<td>8.25±0.64</td>
<td>9.5±2.46</td>
<td>10.25±1.25</td>
</tr>
<tr>
<td>Flagellum with ansa</td>
<td>4.25±0.85</td>
<td>7.5±1.04</td>
<td>1.75±0.47</td>
<td>2.74±0.85</td>
<td>3.5±0.86</td>
</tr>
<tr>
<td>Bent at cephalocaudal region</td>
<td>3.5±0.64</td>
<td>2.5±0.64</td>
<td>1.5±0.28</td>
<td>2.25±0.74</td>
<td>2.75±0.85</td>
</tr>
<tr>
<td>Amorphous</td>
<td>1.25±0.62</td>
<td>1.5±0.65</td>
<td>1.25±0.25</td>
<td>--</td>
<td>0.5±0.28</td>
</tr>
<tr>
<td>Double headed</td>
<td>--------</td>
<td>2.75±0.85</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>Multiple abnormality</td>
<td>--------</td>
<td>15.75±2.68</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>Double tailed</td>
<td>--------</td>
<td>4.0±0.40</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>Hookless flagellum</td>
<td>--------</td>
<td>3.75±1.10</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>Coiled flagellum with microcephali</td>
<td>--------</td>
<td>8.75±0.94</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>Total abnormality</td>
<td>18.5±2.27</td>
<td>52.75±1.75</td>
<td>12.75±1.70</td>
<td>14.5±2.75</td>
<td>17.0±2.98</td>
</tr>
</tbody>
</table>

Values are the means ±SE (N=5).* means P<0.05 when compared with positive control using one way ANOVA followed by Student-Newman-Keuls Test for multiple comparison.

DISCUSSION

Pharmacological characteristics of leave extract of CO-A002 as mentioned in the introductory section are well known in Ayurveda for different ailments like mutagensis, inflammation, diabetic, microbes etc. It is pertinent to deems whether CO-A002 produces genotoxicity in general or any change in the morphology of sperm cell due to the usage of CO-A002 in all age groups for treatment of different diseases. Moreover, it is regulatory requirement that a extract should be evaluated for the genotoxicity potential keeping safety as an important measure. In present study, the leave extract of CO-A002 was found safe. The bone marrow micronucleus test has been the most suitable for the evaluation of genotoxicity along with other tests battery (chromosomal aberration, comet assay and sperm morphology test).

Genotoxic substances possess the ability to induce alteration in the sperm cells morphology because they could modify the normal process of gametogenesis or synchronization of the stages in the seminiferous epithelium. Number of reasons are mentioned for the induction of abnormal sperms in rodents. Probably they are due to the naturally taking place errors in differentiation process or due to the result of an

Figure 6: Comparison of percentage of abnormal sperms found in different treated groups of rat.
abnormal chromosome complements. [23] Autosomes mutation is responsible for the charachersitic changes associated with shape of the sperm head whereas sperm abnormality is caused due to changes in testicular DNA. [24, 25] Sperm mutations are induced by exogenous compounds via point mutations and more likely because of test substance inimical nature on spermatogensis. The results of the study on sperm shape revealed no significant effect on any of the reported abnormalities stating that CO-A002 extract is neither inducing point mutations.

[26] Decrease in the percentage of PCEs and increase in the percentage of MNPCEs and MNNCEs are the indicator of chromosomal aberrations. Such damages or abnormalities are accountable for the appearance of micronuclei at the anaphase of mitosis and hence it is indication of genotoxic to the nuclei. Different doses of CO-A002 extract did not show any significant alteration in the percentage of PCEs, MNPCEs and MNNCEs recommending that such extract is non genotoxic in nature. Whereas the positive control CP-40mg/kg is well known for genotoxic potential and exhibited significant decrease in the PCEs % and increase MNPCEs and MNNEs % thereby proving the genuineness of the experiment.

[27] Comet assay also called as single cell gel electrophoresis assay is a reliable, sensitive and rapid method for detection of DNA damage of the cells, induced by genotoxic substances. This test is based on the embedding of the cells in agarose followed by their lysis in alkaline buffer and at the end a electric current of 300mA 20v for 20 min. is passed. The images known as comets due to their appearance and such images are measured in order to determine damage of DNA. The results of variable doses of extract of CO-A002 depicted in table did not show significant alteration in the OTM value in comparison to the control group and hence proving that CO-A002 extract is safe as far as genotoxicity is concerned, whereas the positive control group HgCl2 (0.864mg/kg) showed highly significant difference in all the observed parameters (Table 3).

In conclusion, battery of tests conducted in present study which shows that CO-A002 leaves extract did not exhibit any alteration in the above mention cytogenetic parameters and explains that it does not possess any genotoxic potential at reported/therapeutic dose levels. This data adds value to the safety of CO-A002 extract which has been already proved by [28] conducting its acute and sub-acute toxicity studies in rodents for its use in human consumption.

REFERENCES:


