Chemopreventive effect of Annona squamosa extract

Singh, Snehal and Agrawal, R.C*

Department of Research, Priyamvada Birla Cancer Research Institute, J.R.Birla Road, Satna - 485005 Madhya Pradesh, India

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Abstract- Annona squamosa is commonly known as custard apple which is eatable. In the folk medicine, the different parts of plant are used for medicinal purposes. Objectives: The aim of this study was to assess the anticarcinogenic, antioxidant and antibacterial effects of Annona squamosa leaves and seeds extract in mouse skin Papilloma model, DPPH method and disk diffusion methods. Materials and Methods: Chemopreventive activity was evaluated by two stage protocol consisting of initiation with a single topical application of a carcinogen (7, 12-dimethylbenz (a) anthracene (DMBA) followed by a promoter (croton oil) two times in a week were employed. The antioxidant and antibacterial effects were assessed by using DPPH and Disk diffusion methods respectively. Results: A significant reduction in tumor incidence, tumor burden and cumulative number of papillomas was observed in mice treated tropically with Annona squamosa extracts as compared to the control group treated with DMBA and croton oil . The antioxidant and antibacterial effect of Annona squamosa extract was also observed. Conclusion: The above studies reveal information about the prevention of cancer. Therefore, the present study is immensely important in future drug development programs for the cancer treatment.

Index Terms- papilloma, Annona squamosa, Antibacterial, antioxidant.

I. INTRODUCTION

Annona squamosa is commonly known as custard apple which is eatable. In the folk medicine, the different parts of plant are used. The leaves are used as a vermicide and are applied to abscesses, insect bites and other skin complaints. Scrapings of root-bark are used for toothache. Powdered seeds are used to kill head-lice and fleas. Annona squamosa (Custard apple) seeds are generally thrown away as waste materials. They are known to possess insecticidal, anti-ovulatory, abortifacient and anti-implantation properties (Vohora et al. 1975; Rao et al.1979; Damasceno et al.2002). In one study the anti-cancer properties of custard apple has been reported due to a class of compounds called acetogenins which are very long chain fatty acids (McLaughlin, 2008) and specific to Annonaceaous species (McLaughlin, 2008). Anti-diabetic properties of Annona spp. appear to be related to stimulation of insulin production and enhanced uptake of glucose by muscles leading to stabilization of blood sugar concentrations (Gupta et al., 2005a). Leaf extracts are effective in lowering blood glucose levels and several reports indicates that Annona squamosa leaf extract can substitute effectively for externally administered insulin (Gupta et al., 2005b) The fruit of Annona spp. have been shown to have anti-microbial activities (Wiert et al., 2005) which showed good anti-bacterial activity of the crude methanol extract of sugar apple fruit, and an isolated diterpene, against Staphylococcus aureus and Streptococcus pneumonia. Custard apple was listed as one of the foods with strong anti-obese activity (Niwano et al., 2009). Beppu et al. (2009) showed that oral administration of ethanol extracts of fresh custard apple fruit potently lowered plasma triglyceride concentrations. Studies report widely differing levels of antioxidants in Annona spp. Studies conducted in India (Kaur and Kapoor, 2005), Taiwan (Chen, et al. 2006; Kaleem et al,2006 & Damsceno et al ). Hole et al. (2006) reported the protective effect of aqueous extract of the fruits on isoproterenol induced myocardial infarction (death of heart tissue) in rats.

II. MATERIALS AND METHODS:

1. Chemicals

7, 12 - Dimethylbenz (a) anthracene (DMBA), croton oil from Sigma Chemicals Co. (St. Louis, MO. USA). The other chemicals were obtained from local firms and were of the highest purity. DMBA was dissolved in acetone at a concentration of 100 ug/100ul and croton oil was diluted in acetone to give a 1% dilution.

Animals

Random bred male Swiss albino mice (7- 8 weeks old), weighing 24 ± 2 gm were used for the experiments. These animals were housed in polypropylene cages in the animal house at temperatures of 22 ± 3°C. The animals were provided with standard mice feed and tap water ad libitum.

Preparation of Annona squamosa extract

Plant material (Annona squamosa) was collected locally and identified and the specimen was authentified at DRI, Chitrakoot (MP), and India. The leaves and seeds were washed, air dried, powdered and extracted separately, with 50 % methanol using separating funnel.

Extract thus obtained were vacuum evaporated to make it in powder form. These extract was again dissolved in DDW just before topicaly application.

Experimental design for Skin Carcinogenesis

The dorsal skin on the back area of the animals was shaven 1 day before the commencement of the experiment and only those animals in the resting phase of the hair cycle were chosen for the study. For induction of tumors a two stage protocol consisting of initiation with a single topical application of a carcinogen (7, 12 - dimethylbenz (a) anthracene (DMBA) followed by a promoter.
(croton oil) two times in a week were employed as per our previous modified method of Berenblum (1975) reported elsewhere . The animals were randomly allocated into 7 groups comprising six mice each. The treatment was provided topically on shaved area

Treatment Groups
Group 1 (Untreated control): No treatment
Group 2 (Vehicle control): 100 μl acetone 2 times /week up to 16 weeks
Group 3 (DMBA Alone): - 100 μg DMBA was dissolved in 100 μl acetone and single application was given.
Group 4 (Croton Oil Alone): - 1 % Croton oil was applied on skin 2 times a week up to 16 week.
Group 5 (Annona sqamosa leaves & seeds extracts Alone): - Annona sqamosa leaves and seeds extract were applied on skin in different mice at the dose of 1000 mg/kg, 2 times a week up to 16 week.
Group 6 (DMBA + Croton Oil): - 100 μg DMBA was dissolved in 100 μl acetone and single application was given afterwards 1 % Croton oil was applied on skin 2 times a week up to 16 week.
Group 7 (DMBA + Annona sqamosa leaves and seeds extract + Croton Oil): -
100 μg DMBA was dissolved in 100 μl acetone and single application was given afterwards the 100 μl dose of Annona sqamosa leaves and seeds extract were applied at different mice at the dose of 1000 mg/kg b. wt. dose was given one hour before the each application of 1% croton oil 2 times a week up to 16 weeks.

The animals of all groups were kept under observation for gross and microscopic changes in skin were observed . During the period of 16 weeks of experimentation, mice of all groups were weighed carefully examined once a week for skin papillomas and these were recorded. The no. of papillomas every weeks were recorded.

Anti-bacterial activities
Antibacterial activities of hydro-methanolic extract from Annona sqamosa leaves was Investigated using the Disk diffusion method given by Kerby-Bauer Disk Diffusion Susceptibility test.

Bacterial strain :
Following gram negative and gram positive bacterial strain i.e. Pseudomonas aeruginosa, Escherichia coli, Shigella flexneri, Staphylococcus aureus, Staphylococcus epidermidis, Bacillus subtilis used for the Antibacterial activities which were received from stock culture of our laboratory.

Media
Nutrient agar broth media were used for the antibacterial activities. Nutrient broth is prepared i.e. 1.3g in 100ml of double distilled water, poured in 6 different test-tubes and added 6 bacterial strain in each test-tube. Nutrient Agar media prepared poured in Petri plates after solidifying swab the bacterial cultures on the plates and allowed for incubation at 37°C for 24 hrs.

Concentration
4 different concentrations of crude extract were prepared (100%, 75%, 50%, 25%).
100%= 1g crude extract in 1ml of double distilled water freshly prepared afterward serial dilution prepared 75%= 75mg in 1ml, 50%= 50mg in 1ml, 25%= 25mg in 1ml.

Study parameter
Measurement of Zone of Inhibition (In mm).

Anti-oxidant activities
Anti-oxidant activities of Annona sqamosa leaves extract (10-100 μg/ml) were determined according DPPH method.

The Formula:
% Inhibition = Abs. 532nm control - Abs. 532nm /control x 100

Study parameter
Results are concluded on the basis of plotting graph = % inhibition of TBARS vs Concentration.

III. RESULTS

Papilloma Test
It was observed that the topical application of Annona Squamosa on Hydromethonlic seed & leaves extract on skin at the dose of 1000 mg/kg body weight have prevented the appearance of papillomas. A significant reduction was observed in tumor incidence, tumor burden, tumor weight, tumor size, cumulative number of papillomas, in Annona Squamosa - treated groups relative to the carcinogen treated control. The papillomas appearance was delayed by about 2 weeks in the animals which received prior application of Annona Squamosa extract as compared to the DMBA + Croton Oil group. The mean no. of papillomas in DMBA+ Annona Squamosa leaves and seed extract + DMBA + Croton oil group was 5 and 2.4 respectively as compared to 6.6 in DMBA + Croton Oil group. The tumour incidence was 83 % and 66 % in leaves and seed extracts as compared to 100 % in control group. (Table 1 -2 & graphs 1-4).

The antibacterial effects of Annona squamosa leaves and seed extract was also observed in gram negative and positive bacteria’s (Table3-4 & graphs 5-6).

The dose dependent antioxidant effect of leaves and seed extracts were also observed in DPPH method (Table 5 & graph 6).

IV. DISCUSSION
The anticarcinogenic nature of Annona squamosa leaves and seeds extract may be due to presence of Acetogenins and flavonoids as Active compounds (Alali et al,1999). The plant was reported for anticancer activity on MCF 7 (epithelial cells of human breast cancer) and A 549 (epithelial cells of human lung cancer) under in vitro conditions by MTT assay (McLaughlin, J. (2008) Annona Squamosa showed predominantly significant anti-inflammatory activity (Mukhlesur 2005). Several studies suggested that compounds possessing anti-inflammatory property inhibit 12-O-tetradecanoyl phorbol-13- acetate induced tumor

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promotion in mouse skin. Aurore et al (1997) reported that anti-inflammatory steroids drastically inhibit the epidermal DNA synthesis and cellular proliferation induced by phorbol ester tumor promoters, a pre-requisite for tumorigenesis. Though the exact mechanism underlying the anti-tumour activity of Annona Squamosa has not been ascertained but it may be inferred that due to anti-inflammatory property of Annona Squamosa might have played a synergistic role in the inhibition of tumorigenesis as observed in the present investigation. In our previous report the anticancer activity of Annona squamosa extract has been reported in mouse skin carcinogenesis model ( Agrawal et al, (2018). The antioxidant and antibacterial activity of Annona extracts have been also observed. The overall results indicates the promising baseline information for the potential uses of the methanol extracts of Annona squamosa leaves as an anti cancer agent.

REFERENCES
[16] Mukhlesur Rahman M, Shahnej Parvin, Ekramul

AUTHORS
First Author – Snehal Singh,, M.Sc. Research scholar,Priyamvada Birla Cancer Research Institute,Satna,M.P.
Second Author – Dr.R.C.Agrawal,Professor,. Priyamvada Birla Cancer Research Institute,Satna,M.P.
Correspondence Author – Dr.R.C.Agrawal, rcagrawal60@yahoo.com

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Table No.1 Effect of *Annona Squamosa* on Hydromethnolic seed & leaf extract on DMBA induced carcinogenesis Swiss albino mice.

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Group</th>
<th>6 Week</th>
<th>10 Week</th>
<th>14 Week</th>
<th>16 Week</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Untreted</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>DMBAAl one</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>Croton Oil Al one</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>4</td>
<td>DMBA+Control</td>
<td>8/6 (1.3)</td>
<td>39/6 (6.5)</td>
<td>37/4 (9.02)</td>
<td>18/2 (9)</td>
<td>100%</td>
</tr>
<tr>
<td>5</td>
<td>DMBA+A.Sl eavel +Croton Oil</td>
<td>9/6 (1.6)</td>
<td>41/6 (6.8)</td>
<td>10/4 (2.5)</td>
<td>30/6 (5)</td>
<td>83%</td>
</tr>
<tr>
<td>6</td>
<td>DMBA+A.SSeed+Croton Oil</td>
<td>6/6 (1)</td>
<td>40/6 (6.6)</td>
<td>30/6 (5)</td>
<td>(2.4)</td>
<td>66%</td>
</tr>
</tbody>
</table>

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Graph No.1 Effect of *Annona Squamosa* on Hydromethnolic seed & leaf extract on DMBA induced carcinogenesis Swiss albino mice.

**Table 2**

Effect of *Annona Squamosa* on Hydromethnolic leaf & seed extract on DMBA induced carcinogenesis Swiss albino mice.

<table>
<thead>
<tr>
<th>S.No</th>
<th>Group</th>
<th>Treatment Doses</th>
<th>Ist Appearance of Papi l l oma (In days)</th>
<th>Cumul ati ve No. of Papi l l oma</th>
<th>Tumor inci den ce (%)</th>
<th>Tumor Burden</th>
<th>Number of Papi l l oma wi th Tumor</th>
<th>Tumor Size ye l d (in mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>I</td>
<td>Vehicle alone (100 µl acetone)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>&lt;2</td>
</tr>
<tr>
<td></td>
<td>II</td>
<td>DMBA alone (100 µg/ 100 µl acetone)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>&lt;4</td>
</tr>
<tr>
<td></td>
<td>III</td>
<td>Croton Oil (100µl of 1% concentration)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

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<www.ijsrp.org>
<table>
<thead>
<tr>
<th>V (N=6)</th>
<th>A. Squamosa leaves extract alone (1000 mg/kg bwt)</th>
<th>0</th>
<th>0</th>
<th>0</th>
<th>0</th>
<th>0</th>
<th>0</th>
<th>0</th>
</tr>
</thead>
<tbody>
<tr>
<td>VI (N=6)</td>
<td>A. Squamosa Seed extract (1000 mg/kg bwt)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>VII (N=6)</td>
<td>DMBA (100 µg) + Croton Oil (1%)</td>
<td>40</td>
<td>35</td>
<td>6/6 (100%)</td>
<td>6.6</td>
<td>6.6</td>
<td>25</td>
<td>8</td>
</tr>
<tr>
<td>VIII (N=6)</td>
<td>DMBA (100 µg/ 100 µl A. Squamosa leaves + Croton Oil (1%)</td>
<td>58</td>
<td>18</td>
<td>5/6 (83%)</td>
<td>5</td>
<td>5</td>
<td>18</td>
<td>4</td>
</tr>
<tr>
<td>IX (N=6)</td>
<td>DMBA (100 µg/ 100 µl A. Squamosa Seed + Croton Oil (1%)</td>
<td>56</td>
<td>21</td>
<td>4/6 (66.6%)</td>
<td>2.4</td>
<td>2.4</td>
<td>12</td>
<td>3</td>
</tr>
</tbody>
</table>

* DMBA = 7,12 dimethylbenz (a)anthracene, CO= Croton oil, A. Squamosa = Annona Squamosa
Graph 1(a) Showing effect of *A. Squamosa* Hydromethanolic leaves and seed extract on cumulative no. of papilloma induced by DMBA+Croton oil.
Graph 2(b) Showing effect of *A. Squamosa* Hydromethanolic leaves and seed extract on tumor incidence (in %) induced by DMBA+Croton oil.

Graph 3(c) Showing effect of *A. Squamosa* Hydromethanolic leaves and seed extract on tumor burden induced by DMBA+Croton oil.

**Antibacterial Activity**

50% methanolic extract of leaves of Annona Squamosa at the different concentration
25%, 50%, 75%, 100% exhibited antibacterial against *E. coli, Klebsiella, Staphylococcus, and Psuedomonous*.

Table no. 3: Antibacterial activity of *Annona Squamosa* leaves against bacterial strains.

<table>
<thead>
<tr>
<th>% Concentration of Seed Extract (Zone of Inhibition mm)</th>
<th>25</th>
<th>50</th>
<th>75</th>
<th>100</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 <em>E. coli</em></td>
<td>13</td>
<td>16</td>
<td>18</td>
<td></td>
</tr>
<tr>
<td>2 <em>Klebsiella</em></td>
<td>16</td>
<td>18</td>
<td>20</td>
<td>22</td>
</tr>
<tr>
<td>3 <em>Staphylococcus</em></td>
<td>14</td>
<td>19</td>
<td>22</td>
<td>23</td>
</tr>
<tr>
<td>4 <em>Psuedomonas</em></td>
<td>15</td>
<td>16</td>
<td>18</td>
<td>20</td>
</tr>
</tbody>
</table>

*name of microorganism*
Graph 4: showing of zone of inhibition of A.S leaves extract against different strains of bacteria.

Table: Antibacterial activity of *Annona Squamosa* Seeds against bacterial strains.

<p>| | | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td><em>E. coli</em></td>
<td>25</td>
<td>50</td>
</tr>
<tr>
<td>2</td>
<td><em>Klebsiella</em></td>
<td>15</td>
<td>16</td>
</tr>
<tr>
<td>3</td>
<td><em>Staphylococcus</em></td>
<td>19</td>
<td>14</td>
</tr>
<tr>
<td>4</td>
<td><em>Pseudomonas</em></td>
<td>16</td>
<td>15</td>
</tr>
</tbody>
</table>

Graph: showing of zone of inhibition of A.S seeds extract against different strains of bacteria.

3. Antioxidant Activity

::: The In vitro Anti-oxidant potential of *A.Squamosa* hydromethonolic leaves and seeds extract have been evaluated using DPPH methods. It was observed that Annona squamosa leaves and seed extract caused dose dependent TBARS inhibition and caused antioxidant activities.

Antioxidant activity:: Table No.4

In vitro Anti-oxidant potential of *A.Squamosa* hydromethonolic leaf extract in the comparison of standard Anti-oxidant.
<table>
<thead>
<tr>
<th>S.No.</th>
<th>Group</th>
<th>Concentration</th>
<th>IC50 Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>A.Squamosa leaves</td>
<td>600 µg/ml</td>
<td>36.85</td>
</tr>
<tr>
<td>2</td>
<td>Ascorbic acid</td>
<td>700 µg/ml</td>
<td>38.51</td>
</tr>
</tbody>
</table>

**IC50** = Concentration at which % inhibition of TBARS is 50%.
Graph: Showing antioxidant activity of *Annona Squamosa* leaves extract in the comparison of standard Ascorbic acid.