POTENTIAL BENEFIT OF ANNONA MURICATA EXTRACT IN COMBATING CISPLATIN INDUCED INJURY OF PAROTID GLAND IN ALBINO RATS

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ABSTRACT

Introduction: Cisplatin is an effective chemotherapeutic agent that is active against malignant salivary gland tumors. However, cisplatin causes various concomitant adverse effects, such as mucostis, hyposalivation, and cytotoxicity which are associated with high morbidity and mortality. Annona muricata a strong antioxidant was considered effective against inflammatory, oxidative stress and free radical-induced damage.

Objective: Investigation of the possible ameliorating effects of Annona muricata against cisplatin induced injury in parotid gland of albino rats.

Material and methods: Thirty adult male Sprague dawley rats weighing from 200 – 250 gm and 2.5-3 months of age were divided into 3 equal groups. Group I: received 6 mg /kg body wt. of saline by i.p. injection. Group II: received 6 mg /kg body wt. of cisplatin once by i.p. injection. Group III: received 6 mg /kg body wt. of cisplatin once by i.p. injection, then after 3 days animals start receiving 200 mg / kg body wt. of annona orally for 40 days. Five animals of each group were sacrificed with over dose anesthesia after 20 and 40 days from the beginning of treatment with anonna. Parotid glands were excised and prepared for routine histological examination using hematoxylin and eosin stain, Caspase 3 immunohistochemistry, enzyme linked immunosorbent assay of catalase enzyme activity and malondialdehyde.

Results: Administration of Annona muricata ameliorate the histological picture and tissue architecture of the parotid glands, decrease the apoptotic effect and the lipid peroxidation induced by cisplatin and increase the level of catalase enzyme.

Conclusion: Annona muricata extract has shown considerable antioxidant ameliorating effect against the undesirable effect of cisplatin, which could be considered for further investigation towards therapeutic application.


Potential benefit of Annona muricata extract in combating cisplatin induced injury of parotid gland in albino rats

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INTRODUCTION

Chemotherapy, plays an important role in the systemic treatment of salivary gland tumors especially the parotid glands which comprise about 80% of all salivary gland tumors followed by submandibular glands and sublingual glands (1). Chemotherapy is generally used as a palliative treatment of symptomatic locally recurrent and/or metastatic disease that cannot be subjected to further surgery or radiation. Many drugs were tested, only few of them were found to be effective, such as 5-fluorouracil, doxorubicin, and Cisplatin (2). 

Cisplatin [cis-diaminedichloro-platinum II (CP)] is one of the most effective and widely used chemotherapeutic agents against many tumors in head and the neck and ovary (3). The mode of action of CP is attributed to its ability to crosslink with the purine bases on the DNA forming adducts; interfering with DNA repair mechanisms, causing DNA damage, it also causes cell cycle arrest in the G2 phase which lead to apoptosis in cancer cells. Other mechanisms of Cisplatin cytotoxicity on tumors cells include destruction of the mitochondria, decreased ATPase activity, and altered cellular transport mechanisms. Cisplatin also induces formation of reactive oxygen species that trigger cell death besides DNA damage (4-6). Although CP has strong chemotherapeutic effects, some undesirable drawbacks may be caused because of its cytotoxicity, and lack of selectivity for cancer cells as it also affects the normal tissues (3).

Receiving chemotherapy can lead to morphologic destruction in salivary gland tissue. These drugs may lead to direct and/or indirect effects on oral mucosa and salivary glands. The mucositis status secondary to the stomato-toxic effects lead to the increase in the mortality rate and cost of treatment in cancer patients. Swallowing dysfunctions, altered saliva production, speech and taste difficulties, oropharyngeal pain, and oral infections are common sequelae of radiation and chemotherapy (7).

So, patients are in need of supplements that could be taken in conjunction with cisplatin to counter act its harmful effect on normal cells to prevent the xerostomia from which they suffer. Recently, there is a new strategy of identifying natural dietary sources and using it as cytoprotectants (8,9).

Annona muricata L (Soursop or Graviola) is an important ever green tropical fruit species from the family of Annonaceae; it has been used in traditional medicine as remedies for various conditions. Plant polyphenols such as curcumin, tannins, flavonoids, terpenoids, have been studied for many decades, to identify its effects against many diseases related to the increase in oxidative stress and free radical-induced cellular damage, such as neurodegenerative diseases, autoimmune disorders, cancer, diabetes and other inflammatory diseases (10). The results revealed that Annona muricata with its content of annonaceae acetogenins has anti proliferative, effect which was associated with cell cycle arrest in the G1 phase (11, 12). The extract could significantly prevent body weight loss during treatment of cancer as a result of increasing in the appetite or through prevention of liver damage and inflammation, it has also anti-inflammatory and immunomodulatory activities (10).

It is also considered to be a strong antioxidant that played a role in protecting DNA from the damage caused by mitigating various reactive oxygen species (ROS). The medicinal plant acquires its protective nature from the presence of antioxidant components (13). The antioxidant or prooxidant strength of phytocompounds depend on several factors like the pH, the chelating capacity, and solubility characteristics, but the main mechanism is hydrogen donation (14, 15). The plant leaves are found to be anti-spasmodic, anti-inflammatory, hypotensive, immunomodulatory and are rich in annonaceous acetogenins. The n-butanolic leaf extracts of this plant was also found to act selectively by normal cells protection.
and cancer cells destruction. A dose of 10 mg/kg Annonacin lead to shrinkage of the tumors induced in murine models comparable to the commercial drugs adriamycin and cisplatin (11,16).

According to the guidelines of OECD annona muricata was considered nontoxic. The aqueous extract of leaves has median lethal dose which is above the consumption level for a human, this is about 211 mg/kg per day, taking into consideration that an average person consumes one cup of tea three times per day. Therefore, for a person to reach the lethal dose of annona he would require to drink more than 71 cups of tea a day (17). These important criterion of antioxidant activity and safety could give a chance for patients suffering from salivary gland tumors treated with chemo therapy to rescue any remaining normal cell from the harmful effect of the treatment on normal cells.

METHODOLOGY

A) Animals

All the experimental procedures were performed in accordance with Mansoura University research ethics committee and according to NIH guide for the care and use of laboratory animals eighth edition 2011, with ethical committee approval number A14091019. The study was conducted in experimental animal study laboratory of Mansoura University. In this study thirty adult male, pathogen free, white albino, Sprague dawley rats weighing from 200–250 gm and 2.5-3 months of age were used. The animals were housed in individual polypropylene cages and kept in 12h light/ dark cycles and controlled conditions (22±3°C temperature, 55-60% relative humidity). All rats had free access to standard pelleted diet of rodents and tap water.

B) Drugs, extract and chemicals

- Cisplatin (cisplatinum, Ebewe, 1 mg/ml) was obtained from Liba Drug Company, (Istanbul, Turkey).
- Annona muricata leave and stem extract of graviola was purchased in a liquid form from Rainforest Pharmacy, Miami, FL33177, USA, Lot number: 07162015PE. According to the manufacturer information, the ingredient of the extract were graviola leaves and stem in pure sugar cane alchol USP50-60%: and osmotic filtered water. The dry herb potency ratio was 1:3.
- All other chemicals used in the experiment were purchased from Sigma Aldrich St Louis, MO, USA.

C) Study design:

The animals were randomly divided into 3 equal groups (10 rats each) as follow:

- **Group I**: control group, 10 animals received 6mg /kg body wt. saline by i.p. injection.
- **Group II**: 10 animals received 6 mg /kg body wt. cisplatin once by i.p. Injection (18).
- **Group III**: 10 animals received 6 mg /kg body wt. cisplatin once by i.p. injection, then after 3 days animals start receiving 200 mg / kg body wt. Annona muricata orally for 40 days (19).

Five animals of each group will be sacrificed with over dose anesthesia (thiopental sodium, 150mg/kg) after 20 and 40 days from the beginning of treatment with Annona. Parotid glands (total 60 glands from both sides) were rapidly excised and then subjected to:

D) Histological evaluation:

The specimens of the right side were fixed in 10% neutral buffered formalin for 24 hours then embedded in paraffin blocks for cutting into 5 microns sections and stained with Hematoxylin and Eosin (H&E) stain (20).
E) Immunohistochemical evaluation

- The neutral buffered formalin fixed specimens were immune-stained using avidin-biotin technique for Caspase 3 immuno stain (Sigma-Aldrich, St Louis, USA) (21).

- Slides were digitized using Olympus® digital camera installed on Olympus® microscope. The resulted images were analyzed on Intel® Core I3® based computer using VideoTest Morphology® software (Russia) with a specific built-in routine for immunostain quantification.

F) Biochemical analysis (enzy me linked immunosorbent assay):

The samples homogenate of the left side glands were formed in 5-10 ml cold buffer (i.e. 50 mM potassium phosphate, pH 7.5, 1 mM EDTA). Then centrifugation at 10000 × g for 15 minutes at 4°C and the supernatant was kept at -80°C till used for analysis of catalase enzyme activity (22) and lipid peroxidation (malondialdehyde, MDA)(23) using colorimetric kit (Sigma- Aldrich, St Louis, USA). The value is expressed as nmol/g tissue.

G) Statistical analysis:

The data resulting from the biochemical and immunohistochemical analysis was collected, tabulated and statistically analyzed using SPSS (statistical package for social sciences) (version 17.0; Spss; Chicago, IL, USA). The data were calculated in the form of Mean ± Standard deviation (SD). In the comparison between the different groups, the significance of difference was tested using one-way ANOVA (analysis of variance) Pairwise comparison between each 2 group was done using Post Hoc Test (Tukey). For all tests, statistical significances accepted for probability (P) values < 0.05.

RESULTS

Hematoxylin and eosin stain result

Control group consisted of densely packed rounded serous acini with narrow lumen and duct structure, the acinar nuclei appeared spherical and basally situated (Fig. 1A). Group II at 20 days and Group II at 40 days showed signs of tissue destruction such as Loss of acinar outline, hydropic degeneration, vacuolization, crescent shape nuclei and large aberrant nuclei. The duct showed disrupted outline with apoptotic nuclei and some eosinophilic areas could be identified, the destruction increased from 20 to 40 days (Fig. 1B,1C). Group III at 20 days showed partial restoration of the acinar outline with nearly normal nuclear pattern, hydropic degeneration still present and the ductal outline is ameliorated (Fig. 1D). Group III at 40 days showed decrease in the hydropic degeneration and preserved acinar and ductal outlines with little cracks between the acini (Fig. 1E).

Caspase3, MDA and catalase results:

The immune expression of caspase 3 was localized in the cytoplasm. Caspase3 and MDA expression increased in group II at 20 days with further increase at 40 days with significant difference compared to control group, after treatment with annon a the caspase 3 and MDA levels decreased significantly in group III at 20 and 40 days in comparison with group II. (Table1&2) (Figure 2&3)

Catalase enzyme expression was high in the control group, in group II the level of the enzyme decreased at 20 and further decrease at 40 with significant difference compared to group I, after treatment with annon a in group III the catalase level begin to increase again at 20 days and increased more at 40 days with significant difference compared to same days at group II. (Table 1&2) (Figure3)
Fig. (1) Photomicrographs of rat parotid glands sections (H& E X 400): (A) Control group consisted of densely packed rounded serous acini with narrow lumen and duct structure, the acinar nuclei appeared spherical and basally situated. (B) Group II at 20 days, (C) Group II at 40 days showed: Loss of acinar outline (black arrow), hydropic degeneration (green arrow), vacuolization (blue arrow), crescent shape nuclei (red arrow), large aberrant nuclei (white arrow). The duct shows disrupted outline (arrow head), apoptotic nuclei (black arrow head) and some eosinophilic areas could be identified (astrics). (D) Group III at 20 days: the gland sections show partial restoration of the acinar outline with nearly normal nuclear pattern (yellow arrow), hydropic degeneration still present (green arrow) and the ductal outline is ameliorated. (E) Group III at 40 days: the hydropic degeneration decreased, the acinar and ductal outlines are preserved (arrow head) with little cracks between the acini (tailed arrow).
Fig. (2) Photomicrographs of rat parotid glands sections showing caspase 3 expression (caspaseX 400): (A) Control group weak expression, (B) Group II at 20 days moderate expression, (C) Group III severe expression, (D) Group III at 20 moderate expression (E) Group III at 40 days weak expression
TABLE (1): Comparison between the studied groups according to Caspase 3, MDA and catalase

<table>
<thead>
<tr>
<th>GROUP</th>
<th>CASPASE 3</th>
<th>MDA</th>
<th>CATALASE</th>
</tr>
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<tbody>
<tr>
<td>I (CONTROL)</td>
<td>0.30±0.0</td>
<td>0.10±0.0</td>
<td>0.30±0.0</td>
</tr>
<tr>
<td>II (20 DAYS)</td>
<td>7.39±3.16</td>
<td>0.23±0.0</td>
<td>0.20±0.0</td>
</tr>
<tr>
<td>II (40 DAYS)</td>
<td>11.11±3.16</td>
<td>0.25±0.0</td>
<td>0.19±0.0</td>
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<tr>
<td>III (20 DAYS)</td>
<td>1.57±0.68</td>
<td>0.15±0.0</td>
<td>0.27±0.0</td>
</tr>
<tr>
<td>III (40 DAYS)</td>
<td>0.57±0.0</td>
<td>0.13±0.0</td>
<td>0.28±0.0</td>
</tr>
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F(P) 28.511* (<=0.001*) 2434.882* (<=0.001*) 1538.101* (<=0.001*)

Data expressed by using mean ± SD. for 5 replica each
Means with Common letters are not significant (i.e. Means with Different letters are significant)
F: F for ANOVA test, Pairwise comparison bet. each 2 groups was done using Post Hoc Test (Tukey)
p: p value for comparing between the studied groups
*: Statistically significant at p ≤ 0.05

TABLE (2): Significant difference between the studied groups according to Caspase 3, MDA and catalase

<table>
<thead>
<tr>
<th>GROUP</th>
<th>CASPASE 3</th>
<th>MDA</th>
<th>CATALASE</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>p&lt;Control</td>
<td>Sig. bet. groups</td>
<td>p&lt;Control</td>
</tr>
<tr>
<td>I (CONTROL)</td>
<td>p&lt;0.060,</td>
<td>p&lt;0.001*</td>
<td>p&lt;0.001*</td>
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<tr>
<td></td>
<td>p&lt;0.002,</td>
<td></td>
<td>p&lt;0.001*</td>
</tr>
<tr>
<td>II (20 DAYS)</td>
<td>&lt;0.001*</td>
<td>p&lt;0.001*,</td>
<td>&lt;0.001*</td>
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<tr>
<td></td>
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<tr>
<td>II (40 DAYS)</td>
<td>&lt;0.001*</td>
<td>p&lt;0.001*,</td>
<td>&lt;0.001*</td>
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<td></td>
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<tr>
<td>III (20 DAYS)</td>
<td>0.855</td>
<td>p&lt;0.001*,</td>
<td>&lt;0.001*</td>
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<tr>
<td>III (40 DAYS)</td>
<td>1.000</td>
<td>p&lt;0.932</td>
<td>&lt;0.001*</td>
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</table>

Data expressed by using mean ± SD. for 5 replica each
Pairwise comparison bet. each 2 groups was done using Post Hoc Test (Tukey)
p: p value for comparing between the studied groups
p<Control: p value for comparing between Control and each other groups
p<1: p value for comparing between Group II (20 days) and Group II (40 days)
p<2: p value for comparing between Group II (20 days) and Group III (20 days)
p<3: p value for comparing between Group II (20 days) and Group III (40 days)
p<4: p value for comparing between Group II (40 days) and Group III (20 days)
p<5: p value for comparing between Group II (40 days) and Group III (40 days)
p<6: p value for comparing between Group III (20 days) and Group III (40 days)
*: Statistically significant at p ≤ 0.05
DISCUSSION

Radio and chemotherapy targeting salivary glands usually results in a variety of debilitating side effects. One direct problem is xerostomia and its subsequent effect of mucositis which was due to extreme sensitivity of salivary glands despite of its low proliferation rate and high differentiation cellular state (24). Most chemotherapeutic agents, such as cisplatin, are assumed to make morphological damage to salivary glands and to worsen its function despite of its potent anti-tumor effect (25, 26).

This study attempted to clarify the adverse effects of chemotherapy on rat’s parotid glands and the possible role of annona muricata to ameliorate these effects. In the current study, injection of cisplatin affected the histological picture of the rat parotid gland. Signs of acinar degeneration appeared represented by disfigured lobular structure and loss of acinar outline, severe vacuolization and disfigured nuclear pattern, these changes increase with time as the glands were more affected after 40 days. These results were in accordance to with Kitashima S (27) (2005) and Oktay et al (28) (2010) who demonstrated that chemotherapeutic agents could induce salivary gland acinar degeneration in the form of acinar and ductal cell vacuolization, neuclear pyknosis and subsequent apoptosis in the cells (27, 28). Intracytoplasmic vacuoles may be caused by the damage of some intracellular structures and fusion of membranes of adjacent secretory granules forming intra-cytoplasmic vacuoles (26). Furthermore these vacuoles could displace the nuclei from its original position forming the noticeable crescent shape that were observed, other explanation suggested that this shape was due to dense areas of chromatin condensation (29).

Sodicoff M, Pratt NE and Sholley MM (30) (1974), termed this shape of the nucleus as light body and described this structure that had complete nuclear envelope and the chromatin segregation so that the heterochromatin was separated from the euchromatin, resulting in crescent form appearance (30). In the pathology of literature the signet ring appearance was originally used to describe cells that exhibit peripheral displacement of the nucleus, the exact etiology of the phenomena is unknown but it may be due to cytoplasmic accumulation of various substances such as mucin or due to cytoplasmic vacuolization related to hydropic degeneration (31).

Homogeneous eosinophilic areas were also seen which was in accordance with Eman hany etal (32) (2017). and Mc Innes (33) (2012), the later indicated that these areas, are thought to accumulation of materials which has proteinaceous nature in the tissues (32, 33). While older studies described them as hyaline degeneration (34).

Cisplatin is activated as soon as it enters the cell. are displaced by water molecules displace the chloride atoms on cisplatin, this hydrolyzed product can react with any nitrogen donor atom on the nucleic acid causing DNA damage, blocking cell division and lead to non-selective apoptosis which affects tumor cells and normal cells as well (35). Caspase staining was relatively high in the cisplatin group at 20 days and its intensity increased at 40 days, it was noticeable that apoptosis occurs in the ducts and acinar cells with higher proportion in the ductal cells which means that the stem cell pool in the ducts may be affected decreasing the regenerative capacity of the gland, this explanation was in accordance with the study that claimed the reduction in saliva production by cisplatin was due to its ability to prevent regeneration of the glandular tissue when progenitor cells are damaged through blocking of aquaporine expression, or by stabilizing DNA strands (36).

Lipid peroxidation is considered to be an early marker of oxidative damage occurring via reactive oxygen species as the cell membrane is the first line of exposure to free radicals before the other cellular components undergo oxidative alteration (35). Moreover, it was reported that the
expression of the antioxidant genes is very low in salivary glands when compared with body fat and mid gut so the salivary glands are more prone to oxidative stress (37). In the present study the level of malondialdehyde was elevated, while the level of catalase enzyme decreased in the cisplatin control group after 20 and 40 days which reflects the decrease in the ability of the cell to make self-defense against the harmful stimuli. These cytotoxic effects of cisplatin on glandular tissues could be associated with its capability of oxidative stress induction, accumulation of reactive oxygen species and subsequent increase in lipid peroxidation and nitric oxide, these cascade events initiate direct strong cytotoxic effects by facilitating the opening of the permeability transition pore complex (38, 39).

Interestingly, annona treated groups showed marked improvement in gland architecture which appeared in the hematoxylin and eosin results and also in the immuno histochemical results by decreasing the percentage of apoptosis in the treated group. The level of the malondialdehyde decreased showing less destruction to the cell membrane accompanied by the increased level of the catalase enzyme denoting increase in the self defense mechanism of the cells which was in accordance to Sanni, Obidoa, Omale (39) 2014 who revealed that annona muricata increased the activity of serum catalase and decreased the level of malondialdehyde which is an endogenous genotoxic product of ROS (39).

This improvement could be attributed to the beneficial antioxidant activity of annona muricata, which could be explained by its high content of polyphenolic enriched fractions which act as antioxidant (39). This effect may be due to its elevated antioxidant capacity to inhibit ROS production by stopping the activation of the cytosolic component of NADPH oxidase, one of the major intracellular sources of ROS, or by neutralizing the free radicals by transferring either electrons or hydrogen atoms (40).

CONCLUSION

- From this study we concluded that annona muricata could have ameliorative effect on the damaged salivary glands subjected to cisplatin.
- Diet rich in antioxidant is very important to protect against many diseases related to oxidative stress damage.

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