Benefits of Morinda citrifolia L. Fruit Extract on Methotrexate Induced Mitochondrial Toxicity – An In Vivo Study in Rat Liver Mitochondria.

Mhatre Bhakti* and Marar Thankamani

School of Biotechnology & Bioinformatics, D.Y. Patil Deemed to be University, Sector 15, CBD, Belapur, Navi Mumbai, Maharashtra, India-400614.

Abstract: Mitochondrial dysfunction has been implicated in the etiology of many drug-induced toxicities. Methotrexate (MTX), an antimetabolite and one of the folic acid antagonists, has widely been used to treat a variety of disorders such as cancer and several autoimmune disorders. It induces oxidative stress which results in many of its ill effects on different target and non-target organs, altering different metabolic pathways and interfering mitochondrial bioenergetics. Here, in our study, we have assessed the toxicity of methotrexate on rat liver mitochondria and its modulation by Morinda citrifolia L. (Noni) fruit extract. MTX significantly affects mitochondrial function, and activity of important mitochondrial enzymes such as Succinate dehydrogenase (SDH), Malate dehydrogenase (MDH) and NADH dehydrogenase. Reduced Cytochrome C – oxidase activity and Reduced glutathione (GSH) levels in MTX treated group (p<0.001) was also observed. An increase in the activity of calcium dependent ATPase (Ca++ dependent ATPase) was observed along with increase in lipid peroxidation (lpx), and significant decrease in mitochondrial protein in MTX treated groups. Liver mitochondria of animals administered with Noni extract did not show any toxicity and combination of aqueous Noni fruit extract and methotrexate showed amelioration of toxicity as compared to methotrexate alone. Our study suggests that Noni can have significant protective effect on liver injury induced by MTX. Nutraceutical supplement like antioxidants found in edible plants such as Noni may be a safe and effective way of alleviating the liver toxicity. Hence, we propose that combination of Noni and methotrexate can be potentially safer combination in cancer therapy.

Keywords: antioxidant; dehydrogenases; lipid peroxidation; methotrexate; mitochondria; reduced nicotine adenine dinucleotide (NADH).
1. INTRODUCTION

Methotrexate (MTX) is a folic acid antagonist widely used in cancer therapy. It is an antimetabolite, acting as a dihydrofolinic acid analogue that binds to the enzyme dihydrofolate reductase (DHFR) and inhibits the synthesis of tetrahydrofolate, necessary for DNA synthesis. It is a well-known chemotherapeutic and immunosuppressive agent that has been used successfully in many rheumatologic, dermatologic and hematologic diseases. It is also used for sarcoidosis, inflammatory bowel diseases, vasculitis and severe refractory asthma. However, its use is limited due to high incidence of serious dose-dependent toxicity, including hepatotoxicity, renal damage, bone marrow suppression and gastrointestinal mucosal inflammation. Mitochondrial impairment could be inadvertent due to toxic effect of drugs on different target and non-target organs, altering different metabolic pathways and interfering in mitochondrial bioenergetics. Mitochondrial dysfunction can have a variety of harmful effects, such as oxidative stress, energy shortage and cell death. The fruits of the plant *Morinda citrifolia* L., known as Noni in Hawaii, have been used as herbal medicines by ancient Hawaiians as remedies for various ailments. Noni juice in particular has been used in the treatment of diabetes, heart disease, high blood pressure, and kidney and bladder disorders. *Morinda citrifolia* L. with its wide range of phytochemicals has been found to be good source of natural antioxidants. Noni juice has become a very popular supplement used by many cancer patients as a source of energy during chemotherapy. However, there is limited information of its efficacy when used in combination with various chemotherapeutic agents. This study presents our attempt in studying therapeutic impact of co-administration of Noni extract on methotrexate induced alterations in mitochondrial function.

2. MATERIALS AND METHODS

### Table 1. Mode of administration of Drug and Extract

<table>
<thead>
<tr>
<th>Groups</th>
<th>Treatment (n=6)</th>
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</thead>
<tbody>
<tr>
<td>Group I (Control)</td>
<td>Vehicle control (saline)</td>
</tr>
<tr>
<td>Group II (MTX 1)</td>
<td>5mg/kg body weight of methotrexate IP twice weekly for 30 days</td>
</tr>
<tr>
<td>Group III (MTX 2)</td>
<td>1mg/kg bw of methotrexate IP twice weekly for 30 days</td>
</tr>
<tr>
<td>Group IV (Noni)</td>
<td>5mg/kg bw of concentrated <em>Morinda citrifolia</em> L aqueous fruit extract orally for 30 days</td>
</tr>
<tr>
<td>Group V (Noni + MTX 1)</td>
<td>5mg/kg bw of <em>Morinda citrifolia</em> L aqueous fruit extract orally + 5 mg/kg bw of methotrexate IP twice weekly for 30 days</td>
</tr>
<tr>
<td>Group VI (Noni + MTX 2)</td>
<td>5mg/kg bw of <em>Morinda citrifolia</em> L aqueous fruit extract orally + 1 mg/kg bw of methotrexate IP twice weekly for 30 days</td>
</tr>
</tbody>
</table>

At the end of the experimental period the animals were anaesthetised using chloroform and killed by cervical decapitation.

2.4 Isolation of rat liver mitochondria

Liver was dissected out, washed with ice cold saline and blotted dry. 300mg of tissue in 2ml mitochondrial isolation buffer was homogenised and final volume was made to 5ml with mitochondrial buffer pH 7. The homogenate was centrifuged at 2000rpm for 10mins at 4°C to remove cell debris. The supernatant was then transferred into fresh tubes and pellet containing cell debris was discarded. The tubes containing supernatant was centrifuged at 11,000 rpm for 10mins to pellet mitochondria. The pellet was washed twice and centrifuged to remove contaminants. The pellet containing mitochondria was reconstituted in 1.15% KCl solution. Purity of mitochondrion was confirmed by assay of succinate dehydrogenase activity. Fresh mitochondria were isolated for each experiment. Protein content was determined by method of Lowry et al. using BSA as standard. Protein content was adjusted 1.0 to 3.5mg/ml for assessment of enzyme activity. Suitable aliquots of mitochondrial suspension containing 0.4-1.0 mg of protein were used for the assay.

2.1 Drugs and chemicals

Methotrexate injection Folitrax -15 IP (15mg/ml) was purchased from IPCA Pharmaceuticals, Mumbai, India. The *Morinda citrifolia* L. fruits were purchased from Abirami Botanicals, Tamil Nadu, India. It was authenticated by the Botany department of St. Xavier’s College, Mumbai as *Morinda citrifolia* L belonging to family Rubiaceae with Blatter Herbarium specimen number 108. The fruits were air dried for 2 days and grounded to fine powder. All other chemicals used were of high analytical grade and solvents were of Qualigen grade. The aqueous extract of Noni was prepared by cold maceration of 250 g of the shade-dried fruit powder in 500 ml of distilled water allowed to stand overnight, and boiled for 5-10 minutes till the volume was reduced to half its original volume. The solution was then cooled, filtered, concentrated, dried in vacuum (yield 36 g) and the residue stored in a refrigerator at 2-8°C for subsequent use.

2.2 Animal model

Adult male albino rats of Wistar strain (100 ± 20 g) were obtained from Bharat Serum Pvt. Ltd, Thane, Navi Mumbai, India. The animals were maintained under temperature (25 ± 2°C), light (12 h light/ 12 h dark) and standard conditions of humidity. Animals were fed standard rat pellets acquired from Lipolin, India along with water ad libitum. Handling of experimental animals were according to the Institutional legislation, structured by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) India (Registration number and date 1553/PO/a/11/CPCSEA Date 04/04/2012).

2.3 Experimental Design

Following the acclimatization period, the rats were randomly divided into groups consisting of six animals’ each as mentioned in Table 1.
2.5 Measurement of complex II activity: Succinate dehydrogenase (E.C. 1.3.99.1)

Succinate dehydrogenase (SDH) assay was carried out using the method described by Slater and Bonner.22 The rate of oxidation reaction was followed by coupling the reaction to the redox dye 2, 6-dichlorophenol-indophenol. The blue DCPIP accepts hydrogen from reduced flavin adenine dinucleotide gets reduced and becomes colourless. Absorbance of the mixture was measured at 600 nm. Enzyme activity was expressed as µmol succinate oxidized/ min/mg protein.

2.6 Assay of Malate dehydrogenase (E.C. 1.1.1.37)

Malate dehydrogenase assay was performed by the method described by Mehler et al.23 Determination of enzyme activity was based on measurement of the rate of oxidation of NADH in the presence of enzyme and excess oxaloacetate. The change in absorbance was monitored at 340 nm for 3 min. Enzyme activity was expressed as µmol of malate oxidized/min/mg protein.

2.7 Assay for complex I activity: NADH dehydrogenase (EC: 1.6.99.3)

NADH dehydrogenase assay was done by the method of Minakami et al.24 The determination of activity was based on the rate of oxidation of NADH measured in the presence of enzyme. Suitable aliquots of mitochondrial suspension were added to the test tubes containing reaction mixture and the change in O.D was measured at 420nm for 3min, at every 30sec interval in UV spectrophotometer. Enzyme activity was expressed as µmoles of NADH oxidised/ min/ mg protein.

2.8 Measurement of complex IV activity: Cytochrome C oxidase. (EC 1.9.3.1)

The assay for cytochrome C oxidase was performed using the method described by Pearl et al.25 Determination of cytochrome c oxidase is based on the rate of formation of water-soluble quinonediimonium red from phenylenediamine. This is proportional to the activity of cytochrome C oxidase. The change in absorbance was monitored after every 30secs for 3 mins at 550 nm in UV spectrophotometer. Enzyme activity was expressed as change in O.D/min/mg protein.

2.9 Estimation of GSH

Reduced glutathione was estimated by the method of Moron et al.26 The absorbance was read at 412nm against blank and expressed as nmoles of GSH/ mg protein.

2.10 Measurement of Ca2+ dependent ATPase (EC 3.6.3.8) activity

The assay was done by the method of Martonosi and Beeler,27 followed by estimation of phosphorus by the method of Frisk and Subbaraw.28 Enzyme activity was expressed as µmoles of inorganic phosphorous liberated/min/mg protein.

2.12 Estimation of total mitochondrial protein

Estimation of total mitochondrial protein was estimated by the method of Lowry et al.29

3. STATISTICAL ANALYSIS

Data are presented as Mean ± SD. The significance of difference among the groups was assessed using one way analysis of variance (ANOVA) SPSS 14 followed by student t test. In figures and tables, symbols represent statistical significance as indicated: *p< 0.05, **p< 0.01, ***p<0.001 and NS- Non-significant.

4. RESULTS

In the present study, we have tried to understand effect of MTX on rat liver mitochondrial enzymes under influence of Noni. Effect on mitochondrial dysfunction can be measured by alterations in the activities of mitochondrial enzymes as presented in Table 2.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Group I Control</th>
<th>Group II MTX1</th>
<th>Group III MTX 2</th>
<th>Group IV Noni</th>
<th>Group V Noni + MTX 1</th>
<th>Group VI Noni + MTX 2</th>
<th>ANOVA P -Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>SDH µmoles of Succinate oxidized min/mg protein</td>
<td>9.5±0.26</td>
<td>5.3±0.29**</td>
<td>7.31±0.21**</td>
<td>9.2±0.71</td>
<td>7.6±0.32**</td>
<td>8.7±0.21**</td>
<td>0.001</td>
</tr>
<tr>
<td>MDH µmoles of Malate oxidized min/mg protein</td>
<td>0.32±0.04</td>
<td>0.16±0.41**</td>
<td>0.22±0.03**</td>
<td>0.36±0.03d</td>
<td>0.25±0.03d</td>
<td>0.34±0.03**</td>
<td>0.001</td>
</tr>
<tr>
<td>NADH µmoles of NADH oxidized/min/mg protein</td>
<td>0.21±0.007</td>
<td>0.15±0.02**</td>
<td>0.18±0.019**</td>
<td>0.23±0.01e</td>
<td>0.16±0.018d</td>
<td>0.20±0.008e</td>
<td>0.001</td>
</tr>
</tbody>
</table>

Values are expressed as Mean ± SD for (n=6) six rats in a group. Statistical significance was tested with ANOVA and complemented with Student’s t-test; Values carrying different superscripts are significantly different. Comparisons are expressed as ‘a’ Group I and Group II, ‘b’ Group I and Group III, ‘c’ Group I and Group IV, ‘d’ Group II and Group V, ‘e’ Group III and Group VI’. Statistical significance represented as: *p < 0.05, **p < 0.01, ***p < 0.001, NS- Non-significant.
Activity of SDH significantly decreased \((p < 0.001)\) in Group II MTX 1 and Group III MTX 2 treated rat liver mitochondria, but activity of SDH in Group IV Noni treated animals was akin to that of control (Table 2). Liver mitochondria of animals treated with both MTX and Noni in Group V and VI has reduced activities of SDH as compared to control and Noni treated group. Group VI Noni +MTX 2 was found to have maintained activities of SDH to some extent. Reduction in other TCA cycle and ETC enzyme activities of Group II (MTX 1) and Group III (MTX 2) further confirms the mitochondrial toxicity of MTX, however, the effect of MTX on Group III animal was lesser and this could be due to low dose of MTX drug in Group III treated animals (Table 2). Lipid peroxidation product may also contribute to modification of malate dehydrogenase leading to a decrease of enzyme activity in drug-treated group as in (Table 2). Activities of these enzymes in Noni treated group were maintained similar to that of control. Co-supplementation of Noni in Group V and VI maintained enzymatic activities to some extent indicating the possible role of oxidative stress in MTX-induced mitochondrial dysfunction and the antioxidant effect of Noni.\(^{30}\) However, since the dose of MTX in Group V was more as compared to that of Group VI, Group VI showed lesser degree of alterations as compared to that of Group V (Table 2). According to our study MTX might have reduced availability of NADPH in mitochondria, which might have affected electron transfer in ETC. Dihydrofolate reductase is an NADPH-dependent oxidoreductase that oxidizes NADPH to NADP\(^+\) whilst reducing dihydrofolate acid to tetrahydrofolate acid \(^{31}\) thus NADPH is vital in maintaining its activity. MTX treated Group II and III animals had reduced activities of NADH dehydrogenase in mitochondria as compared to the control (Table 2). Animals treated with Noni alone in Group IV were found to have maintained activities of all enzymes near normal, demonstrating nontoxic nature of Noni. Animals treated with MTX, co-supplemented with Noni (Group V) had reduced activity as compared to control but where significantly improved in comparison to that of Group II and Group III. Group V (Noni + MTX 2) had activity similar to that of control indicating - lesser dose of MTX might have reduced the adverse effects (Table 2). Reduced cytochrome C–oxidase activity in MTX II than in MTX I suggests dose dependent inner membrane damage (Table 3) which can be attributed to loss of protein content due to peroxidative attack on its unsaturated fatty acid by oxygen free radicals.\(^{32}\) Group IV Noni had non-significant alterations in the activity of cytochrome C –oxidase when compared to control indicating no mitochondrial toxicity.

### Table 3. Effect of Noni on MTX mediated changes on cytochrome C oxidase activity and GSH levels.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Group I C</th>
<th>Group II MTX 1</th>
<th>Group III MTX 2</th>
<th>Group IV Noni</th>
<th>Group V Noni + MTX 1</th>
<th>Group VI Noni + MTX 2</th>
<th>ANOVA P–Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytochrome C oxidase change in OD min/mg protein</td>
<td>0.049±0.003</td>
<td>0.039±0.005(^{***})</td>
<td>0.043±0.003(^{***})</td>
<td>0.049±0.003(^{***})</td>
<td>0.042±0.002(^{***})</td>
<td>0.046±0.004(^{***})</td>
<td>0.001</td>
</tr>
<tr>
<td>GSH nmoles of GSH/mg protein</td>
<td>99.21±5.99</td>
<td>41.58±5.20(^{***})</td>
<td>81.27±6.3(^{***})</td>
<td>93.52±0.13(^{***})</td>
<td>78.42±4.9(^{***})</td>
<td>99.90±5.6(^{***})</td>
<td>0.001</td>
</tr>
</tbody>
</table>

Values are expressed as Mean ± SD for (n=6) six rats in a group. Statistical significance was tested with ANOVA and complemented with Student's t-test; Values carrying different superscripts are significantly different. Comparisons are expressed as 'a'Group I and Group II, 'b'Group I and Group III, 'c' Group I and Group IV, 'd'Group II and Group V, 'e'Group III and Group VI. Statistical significance represented as: * \(p < 0.05\), ** \(p< 0.01\), *** \(p < 0.001\), NS- Non-significant.

Liver is the main site for synthesis of GSH and detoxication of different xenobiotics and drugs. There is a significant reduction in mitochondrial GSH levels in MTX treated groups as also reported by Sener, et al.\(^{33}\) So reduced NADPH can be one of the reasons for reduced GSH levels in MTX treated groups \((p<0.001)\) (Table 2). Groups administered with combination of Noni and MTX, had significantly improved levels of GSH than MTX alone (Table 3) suggesting drugs can cause adverse effects which can be regulated by prophylactic activity of herbal drugs as has been reported by Chavez et al.\(^{34}\) \(\text{Ca}^{2+}\) ATPase activity enhanced significantly in MTX treated Group II and III (Fig. 1). \(\text{Ca}^{2+}\) uptake may increase \(\text{O}_2\) production in MTX treated Group II and III. Excessive amount of \(\text{Ca}^{2+}\) can cause mitochondrial damage.
Values are expressed as Mean ± SD for (n=6) six rats in a group. Statistical significance was tested with ANOVA and complemented with Student’s t-test; Values carrying different superscripts are significantly different. Comparisons are expressed as 'a' Control and MTX 1, 'b' Control and MTX 2, 'c' Control and Noni, 'd' MTX I and Noni +MTX 1, 'e' MTX 2 and Noni +MTX 2. Statistical significance represented as: * p < 0.05, ** p< 0.01, *** p< 0.001, NS- Non-significant.

Fig 1. Effect of Noni on MTX mediated changes in activity of mitochondrial Calcium dependent ATPase.

This flux can potentially alter intracellular Ca^{2+} signals. Activity of Ca^{2+} ATPase in mitochondria of animals treated with Noni (Group IV) showed no change (Fig. 1) as compared to that of control hence suggesting no toxic effect. Fig. 2 shows the levels of lipid peroxides in mitochondria of methotrexate and Noni treated animals. The oxidative stress generated by MTX might have led to increase in lipid peroxide levels in Group II and III. MTX is proxidant in nature as also reported by Moghadam et al and Ozogul et al. Antioxidants have an ability to retard this effect which otherwise can lead to many chronic diseases.

Values are expressed as Mean ± SD for (n=6) six rats in a group. Statistical significance was tested with ANOVA and complemented with Student’s t-test; Values carrying different superscripts are significantly different. Comparisons are expressed as 'a' Control and MTX 1, 'b' Control and MTX 2, 'c' Control and Noni, 'd' MTX I and Noni +MTX 1, 'e' MTX 2 and Noni +MTX 2. Statistical significance represented as: * p < 0.05, ** p< 0.01, *** p< 0.001, NS- Non-significant.

Fig 2. Effect of Noni on MTX mediated changes in mitochondrial lipid peroxide (LPx) levels.
Animals treated with MTX 1 in Group II had elevated levels of MDA due to higher dose than MTX 2 Group II. Reduction in MDA levels can be observed in Group V and VI in comparison to animals treated with MTX alone. Animals treated with Noni alone (Group IV) had MDA levels similar to that of control. After treatment with MTX, protein levels where found to have drastically depleted in Group II MTX 1 treated animals, as compared to control. Group III MTX 2 (Fig 3) also showed decrease but lesser as compared to Group II. This could be due to damage to liver, protein loss and dietary deficiency owing to lack of feed intake. Animals treated with Noni alone (Group IV) had comparable protein content as that of control.

Fig 3. Effect of Noni and MTX on levels of mitochondrial total proteins.

Values are expressed as Mean ± SD for (n=6) six rats in a group. Statistical significance was tested with ANOVA and complemented with Student’s t-test; Values carrying different superscripts are significantly different. Comparisons are expressed as 'a' Control and MTX 1,'b'Control and MTX 2,'c' Control and Noni, 'd' MTX 1 and Noni +MTX 1, 'e' MTX 2 and Noni +MTX 2. Statistical significance represented as: * p < 0.05, ** p< 0.01, *** p< 0.001.

Co-supplementation of Noni with MTX had a very significant impact on the animals treated in Group V (Noni +MTX 1), had more protein content as compared to MTX 1 alone. And the animals treated in Group VI Noni + MTX 2 had protein values similar to that of control and Group IV Noni.

5. DISCUSSION

Mitochondria are dynamic organelles playing a central role in numerous cellular functions including major bioenergetic processes and intracellular calcium homeostasis. They are also involved in formation of ROS and activating programmed cell death or apoptosis. Mitochondrial dysfunction strongly alters its structure and function endangering cell life due to drug induced toxicities. Mitochondrial damage by drugs can result in amplified production of oxygen free radicals, mainly superoxide (O$_2^-$) leading to liver injury. It can also lead to hepatic necrosis or apoptosis which leads to liver failure.

The mitochondrial permeability transition (MPT) is a mechanism which can be used to comprehend the toxic nature of drugs, which can induce cytopathic hepatitis. Drugs can also induce membrane permeabilization through MTP independent mechanism. SDH is an inner mitochondrial membrane protein marker which links the Krebs cycle with the electron transport chain and oxidative phosphorylation. Electron introduced by SDH in ETC at complex II of the respiratory chain is bound to inner mitochondrial membrane and decrease in the activity of SDH can be correlated due to loss of membrane integrity. Co-treatment with Noni reversed the alterations brought about by MTX suggesting that Noni has a membrane stabilizing property. MDH is an oxidoreductase and an extremely stereospecific NAD requiring enzyme which explicitly catalyses a vital reaction in TCA cycle. MTX inhibits many NAD(P)-dehydrogenase enzymes including malate dehydrogenase. Among five complexes, containing different protein and non-protein machinery Complex I or NADH dehydrogenase is one of the largest, playing an essential role of passing electron from NADH to cytochrome Q which is linked by flavin dehydrogenase. Cells capacity to deal with oxidative stress is inhibited due to depletion in NADH and the cellular ability to regenerate glutathione. Cytochrome C oxidase is an important, multisubunit enzyme, which forms an integral membrane protein found nearly in all aerobic organisms on Earth. It catalyses the final step of the electron transfer chain, thereby causing reduction of oxygen to water, at the same time translocating protons across the membrane helping to generate the electrochemical gradient for direct ATP synthesis. The interplay between membrane lipids and cytochrome C oxidase has been studied extensively and it has been repeatedly demonstrated that specific lipid interactions impact its function. Deterioration in cytochrome C oxidase activity can lead to obstruction of electrons, leading to abridged potential of few electrons which can favour auto oxidation; this leakage of electrons can generate superoxide. Iron that is extremely susceptible to ROS, are also present in cytochromes, which may encourage metal-catalysed oxidation within the active centres of respiratory chain complexes. A chain reaction linking the protein to peroxides may consequently, occur within the enzyme complexes, leading to inactivation of proteins. Restriction in the activity of cytochrome C oxidase can hinder transfer of electrons through complex IV. GSH is maintained in reduced state by NADPH and there are reports of a MTX synergistically
inhibiting several enzymes directly involved in maintenance of the cellular redox state via the regeneration of glutathione. Homeostasis of calcium within the mitochondria is maintained by Ca\textsuperscript{2+} ATPase uniporter, which principally maintains inflow of Ca\textsuperscript{2+} and controls mitochondrial Ca\textsuperscript{2+} flux.\textsuperscript{52} Ca\textsuperscript{2+} can elevate ROS production in mitochondria of hypoxic tissue. Severe ATP diminution can inhibit calcium extrusion from the cells thus causing its intracellular accumulation, which in turn can activate endonucleases and proteases leading to destruction and disorganisation of cell membrane thus leading to necrosis.\textsuperscript{53}Devi et al.\textsuperscript{4} also suggested protective effect of P. tomentosa pre-treated rats mitochondrial toxicity which could also be due to presence of anti-oxidant compounds similar to those found in Noni. Drop in proteins content reflects toxic effects of MTX in biosynthetic activity of various tissues.\textsuperscript{55}

6. CONCLUSION

Our study suggests that Noni can have significant protective effect on liver injury induced by MTX. Dietary intervention with antioxidants found in edible plants like Noni may be a safe and effective way of alleviating the toxicities of anticancer chemotherpay. There are many reports of Noni itself showing promising anticancer property. Hence, combinations of cytotoxic anti-tumour agents and phytochemicals can act together producing inhibitory mechanisms on cancer growth and protect normal tissue. This combination strategy shows promise on cancer therapy.

7. AUTHOR CONTRIBUTION STATEMENT

Dr. Thankamani Marar designed the experimental work, reviewed the manuscript, and improved the quality of the manuscript. Dr. Bhakti A Mhatre carried out all laboratory work, data collections, and writing the manuscript.

8. CONFLICT OF INTEREST

Conflict of interest declared none.

9. REFERENCES


AUTHOR CONTRIBUTION STATEMENT

Conflict of interest declared none.


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