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Original Article

Computational Modelling of Three-phase Stent-based Delivery

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Abstract

Background and Objective: Treatment of arterial lesions using drug-eluting stent is now a gold standard method, however, the mechanisms of drug uptake and its retention is not well understood. In most of the computational studies, only the binding of drug to specific receptor is considered; but it is well established that when the drug binds to the specific receptor, there is also occurrence of non-specific binding caused by the trapping of drug in the extracellular matrix. When non-specific binding is not subtracted from total binding to give receptor-mediated binding, a description of receptor/ligand requires that effects of non-specific binding be considered.

Methods: We construct a computational model of the drug transport within the arterial wall. The governing equations, along with the suitable boundary conditions, are solved numerically in an explicit manner. Necessary stability criteria have been checked in our in-house FORTRAN code.

Results: The simulated results in this study predict that the penetration length of both free and non-specific bound drug increases with increase in time, and ultimately saturation of binding sites takes place; however, specific bound drug becomes totally absorbed at the adventitial boundary. The concentration of free drug is always higher in the case of the single bound phase model than that of the double bound phase model.

Conclusion: Because local concentration of free drug is inextricably linked to the binding and saturation of binding, our results provide a potential explanation for the success of stent-based delivery.

Introduction

The drug-eluting stent (DES) is now a common treatment for atherosclerosis. Placed into the narrowed artery, it serves to reopen the artery and slowly release drug to inhibit cell proliferation. The DES consists of three parts, namely the stent platform, a polymer coating that binds the drug to the stent and releases drug, and the drug. Success of the DES is usually associated with the effective delivery of potent therapeutics to the target site, at a sufficient concentration for a sufficient time and in a biologically active state. Although DESs are now the primary choice for treatment of atherosclerosis, questions still arise in regard to longevity and safety.[1]

To investigate the drug release mechanism, various models have been developed, including the one-dimensional model,[2–4] the two-dimensional or axi-symmetric model and the three-dimensional model.[5–13] The drug release process is also affected by some physical properties, namely the geometrical design of the stent itself, the mechanical properties of the stent's material and the chemical properties of the drug.[14,15] A number of experimental and numerical studies on DES have been published in recent years to address such issues as longevity and safety.[3,11,16–22]

Since binding of the ligand to the cell surface receptors has been amenable to direct experimental investigation for roughly the past three decades,[23,24] a sincere attempt has also been made to take into account the binding event that occurs on the cell surface. However, no such attempt has been made to consider the aspects of trafficking process, cell signaling or receptor mobility. The first mathematical model developed to investigate binding kinetics used a constant partition coefficient.[25] But, it was too simple to predict the distribution of drug or to consider retention of the drug in the arterial wall. Some authors used a chemical reaction to explain the binding kinetics and also to relate the association and dissociation coefficients by equilibrium coefficients.[26,27] A nonlinear reversible chemical reaction is generally accepted to predict the two types of drug forms.[28]

Vo and Meere[29] considered a nonlinear reversible binding mod-

Keywords: Embedded drug-eluting stent**;** Free drug**;** Specific binding**;** Non-specific binding**;** Nonlinear saturable reversible chemical reaction.

Abbreviations: DES, drug-eluting stent; SR, specific receptor; ECM, extra cellular matrix; NDC, normalised drug concentration; NDC_{FREE}, normalised free drug concentration; NDC_{SR}, normalised bound drug concentration in SR phase; NDC_{ECM}, normalised bound drug concentration in ECM phase.

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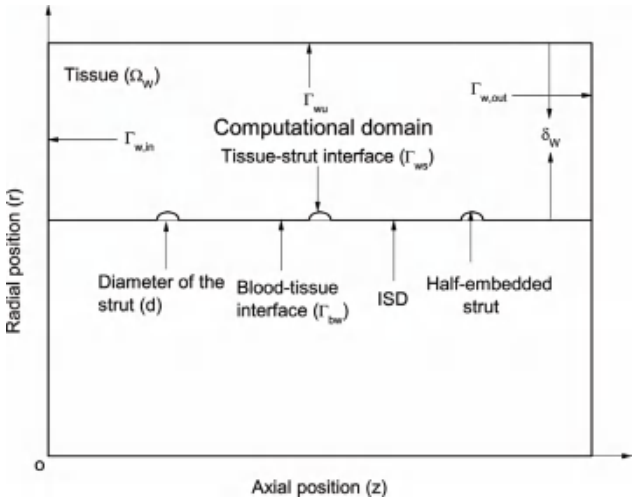

Fig. 1. Schematic diagram of the stented artery.

el to describe the release of heparin-binding growth factors from an affinity-based delivery system. Groh *et al*[30] adopted a nonlinear reaction model to describe the binding of drug to binding sites within cells and also investigated the chemotherapeutic interaction with the microenvironment of cells in tumour drug delivery. A nonlinear saturable binding model was developed to describe the drug binding in arterial tissue sites, which included two phases of drug: the free drug and the bound drug.[18,31]

Different binding models namely, nonlinear saturable reversible binding, nonlinear saturable irreversible binding, linear reversible binding and linear irreversible binding, are demonstrated by McGinty and Pontrelli.[32] However, it is well established that when the drug binds to the specific receptor (SR), there is also occurrence of non-specific binding caused by the trapping of drug in the extracellular matrix (ECM). Tzafriri *et al*[33] investigated two types of binding–specific binding and non-specific binding–and both of the binding kinetics are modelled by using a nonlinear reversible chemical reaction process. Very recently, McGinty and Pontrelli[34] investigated the importance of modelling the specific and non-specific saturable binding in the arterial wall as separate phases.

Very little of this type of work has been carried out considering the specific and non-specific binding of drug,[33,34] and some significant aspects remain unanswered. Tzafriri *et al*[33] considered only the transmural transport of eluted drug with constant release kinetics, whereas, McGinty and Pontrelli[34] opined that when the ratios of SR to ECM binding site density and of SR to ECM unbinding rate are both small, drugs such as sirolimus and paclitaxel have same modes of action.

Usually, modelling with well-apposed DES represents very early stage after the implantation. The scientific interest for embedment of strut came from the need for accurate and quantitative evaluation of the vessel wall and stent interaction. The degree of stent embedment could be one of the surrogate parameters of the vessel wall-stent interaction.[35] In terms of shear stress, the deeper the struts are embedded, the less disturbed the shear stress will be.[36] Moreover, deeper penetration into the vessel wall increases direct contact and drug delivery, and reduces recirculation zones, which, in turn, decreases the area of the endothelisation exposed to disturbed flow, thus increasing the probability of endothelisation of adjacent tissues; ultimately, an antiproliferative and anticoagulant flow environment is established, which is the optimal condition for clinical success.[35]

There seems to be a down side, however, in the sense that embedded struts denote penetration of the cutting edge of the struts through vessel wall, implying larger injury of the vessel that can trigger neointimal hyperplasia.[37] Even if the embedment of struts is small, a larger width of strut could contribute to larger amount of vessel wall-stent interaction. Hence, relationship between this injury (*i.e.* the degree of embedment and neointimal hyperplasia) will be the topic of further study.[38] On that theoretical basis, we should not expect an excess of neointima, despite the embedment and injury to the vessel wall.

Keeping all the relevances in mind, the present investigation describes three-phase drug transport phenomena within the arterial wall, namely free drug, specific binding of drug with the SR and non-specific binding of drug in the ECM site from a half-embedded DES. The transport of free drug has been modelled by reaction-diffusion process, whereas the bound drug has been modelled by nonlinear reversible saturable chemical reaction. The arterial wall has been considered as a single homogeneous layer with identical diffusivity property. In this study, the therapeutic domain of length L consists of three struts with centre to centre distance of 0.7 mm (Fig. 1).[39,40] At the strut surfaces, a time-dependent drug release kinetic in exponential manner has been accounted for McGinty *et al*.[41] The governing equations together with their physiological realistic boundary conditions are solved numerically in an explicit manner.

Governing equations and boundary conditions

A three-phase model of drug transport has been modelled in the tissue, namely free drug,[16] specific binding (SR) and non-specific binding (ECM).[42,43] The transport of free drug has been modelled by reaction-diffusion process and binding of drug by nonlinear saturable reversible chemical reaction. Now the dimensionless governing equations for the model considered may be written as

$$\frac{\partial c_f}{\partial t} = \frac{1}{f^2}\left[\frac{\partial^2 c_f}{\partial r^2} + \frac{1}{r}\frac{\partial c_f}{\partial r} + f^2\frac{\partial^2 c_f}{\partial z^2}\right] - \frac{\partial c_{SR}}{\partial t} - \frac{\partial c_{ECM}}{\partial t}, \quad (1)$$

$$\frac{\partial c_{SR}}{\partial t} = Da_{SR}\left[c_f\left(c_{SR}^{\max} - c_{SR}\right) - \frac{c_{SR}}{k_{eq_{SR}}}\right], \quad (2)$$

$$\frac{\partial c_{ECM}}{\partial t} = Da_{ECM}\left[c_f\left(c_{ECM}^{\max} - c_{ECM}\right) - \frac{c_{ECM}}{k_{eq_{ECM}}}\right], \quad (3)$$

where c_f, c_{SR} and c_{ECM}, normalised by initial strut drug concentration (c_0), are the normalised concentration of free drug, specific and non-specific binding drug respectively. r is the dimensionless radial coordinate and z is the dimensionless axial coordinate, normalised by the thickness of the arterial wall (δ_W) and diameter of the strut (d) respectively. Here, $c_i^{\max}$ is the dimensionless local density of binding site, normalised by initial strut drug concentration(c_0). $Da_i = \frac{k_{f_i} c_0 d^2}{D_i}$ and $k_{eq_i} = \frac{c_0 k_{f_i}}{k_{r_i}}$ are the Damköhler number and equilibrium constant respectively, in which k_{f_i} and k_{r_i} are the association and dissociation reaction rate respectively. Here and in the sequel, i=SR, ECM. Here $t = \frac{tD_f}{d^2}$, is the dimensionless time and $f = \frac{d_w}{d}$.

In the tissue, symmetry boundary conditions are applied on the proximal ($\Gamma_{w,in}$) and the distal ($\Gamma_{w,out}$) walls:

$$\frac{\partial c_f}{\partial z} = 0 \text{ on } C_{w,in} \text{ and } C_{w,out} \quad (4)$$

At the perivascular wall, a perfect sink condition for free drug is applied:

$$c_f = 0 \text{ at } \Gamma_{wu} \quad (5)$$

A time-dependent drug release kinetic has been used at the strut surfaces:[41]

$$c_f = e^{-\varphi t} \text{ on } \Gamma_{ws}, \tag{6}$$

where $\{ = \frac{md^2}{D_i}$, λ is the rate of drug release from strut surfaces.

At the blood-tissue interface (Γ_{bw}), it is assumed that the transported drug is insensitive to luminal flow, which is modelled as zero-flux condition given by

$$\frac{2c_f}{2r} = 0 \text{ on } C_{bw.} \tag{7}$$

Radial coordinate transformation

In order to avoid interpolation error, we transform the therapeutic domain (Fig. 1) into a rectangular one, by making use of the following radial coordinate transformation:

$$p = 1 + \frac{r - R_{tl}}{C_{wu} - R_{tl}} \text{ where } R_{tl} = C_{bw}\ ,\ C_{ws.} \tag{8}$$

Considering this transformation, the irregular domain transforms into [0,*L*]×[1,2]. Now using the transformation (8) the equations (1)–(7) are transformed as follows:

$$\frac{2c_f}{2t} = \frac{1}{f^2}\cdot\frac{2^2 c_f}{2p^2}\cdot\frac{1}{R^2} + f^2\left(\frac{2p}{2z}\right)^2 1 + \frac{2c_f}{2p}\left(\frac{1}{R''R\wedge p-1\,h + R_{tl}}\cdot + f^2\frac{2^2 p}{2z^2} + \frac{f^2}{R^2}\frac{dR}{dz}\,a\wedge p-1\,h\frac{dR}{dz} + \frac{dR_{tl}}{dz}\right)2F + \frac{2^2 c_f}{2z^2} + 2\frac{2p}{2z}\frac{2^2 c_f}{2p2z} - \frac{2c_{SR}}{2t} - \frac{2c_{SR}}{2t}, \tag{9}$$

$$\frac{2c_{SR}}{2t} = Da_{SR}\,\&c_f\,\wedge c_{SR}^{\max} - c_{SR}\,h - \frac{c_{SR}}{k_{eq_{sr}}}0, \tag{10}$$

$$\frac{2c_{ECM}}{2t} = Da_{ECM}\,\&c_f\,\wedge c_{ECM}^{\max} - c_{ECM}\,h - \frac{c_{ECM}}{k_{eq_{ECM}}}0, \tag{11}$$

$$\frac{2c_f}{2z} = 0 \text{ on } z = 0 \text{ and } z = L, \tag{12}$$

$$c_f = 0 \text{ at } \xi = 2, \tag{13}$$

$$c_f = e^{-\varphi t} \text{ at } \xi = 1 \text{ and } z \in \Gamma_{ws} \tag{14}$$

$$;\frac{2c_f}{2p}E_{p=1} = 0 \text{ for z } !\ C_{bw} \tag{15}$$

Method of solution

The governing equations with the set of initial and boundary conditions are solved numerically by finite-difference scheme. Forward-time centred-space discretisation technique has been made use of in the explicit numerical scheme. Let us describe our finite-difference method in more detail: We denote $z_i = i\delta z$, $t^n = n\delta t$, $\xi_k = k\delta\xi$, where *n* refers to the time level, δ*t* the time increment and δ*z* is the space step size along the axial direction. Here, δξ denote space step sizes along the radial direction.

The finite difference approximations of (9–11) are as follows:

$$\frac{c_{f,k}^{n+1} - c_{f,k}^{n}}{dt} = \frac{1}{f^2}\,6diffc_f@_{i,k}^{n} - \frac{c_{SR_{i,k}}^{n+1} - c_{SR_{i,k}}^{n}}{dt} - \frac{c_{ECM_{i,k}}^{n+1} - c_{ECM_{i,k}}^{n}}{dt}, \tag{16}$$

$$\frac{c_{SR_{i,k}}^{n+1} - c_{SR_{i,k}}^{n}}{dt} = 6reac_{SR}@_{i,k}^{n}, \tag{17}$$

$$\frac{c_{ECM_{i,k}}^{n+1} - c_{ECM_{i,k}}^{n}}{dt} = 6reac_{ECM}@_{i,k}^{n}, \tag{18}$$

where the expressions for $6diffc_f@_{i,k}^{n}$, $6reac_{SR}@_{i,k}^{n}$, $6reac_{ECM}@_{i,k}^{n}$ are included in the Supplementary Term S1.

Now, the discretised equations are solved numerically in an explicit manner. No standard package has been used in the present simulation, rather the computational code has been successfully programmed using FORTRAN language.

Results and discussion

For the purpose of numerical computation of the quantities of significance, the computational domain has been confined to a finite nondimensional length of 25 and solutions are computed with the grid size of 501×61 by making use of the input values presented in Table 1.[11,16,31,33,34,39–41,44–46] The simulation concerning grid-independent study was performed for examining the error associated with the grid sizes used and displaced in Figure 2. The transmural variations of normalised bound drug concentration in SR phase at an axial distance of *z*=11 for three distinct grid sizes almost overlap on one another, which clearly establishes the correctness of the grid sizes used.

Transmural variations of free (NDC_{FREE}), bound in ECM phase (NDC_{ECM}) and bound in SR phase (NDC_{SR}) at three distinct nondimensional times have been depicted in Figure 3, panels a–c, respectively. Drug enters the arterial wall at ξ=1 in the free phase, and is rapidly bound to both ECM and SR sites. It is worthy to note that the penetration length of both free and ECM-bound drug increases with increasing time, with saturation of binding sites ultimately taking place. Moreover, the SR-bound drug is absorbed at the adventitial boundary (ξ=2) with the increase of time. The free drug (NDC_{FREE}) and ECM-bound drug(NDC_{ECM}) penetrate half of the thickness of the tissue at *t*=0.5, and although the NDC_{FREE} and NDC_{ECM} profiles are similar, drug concentrations within ECM-bound phase are an order of magnitude greater than for the SR-bound phase. Saturation of SR-bound drug takes place with increasing time (Fig. 3c). All the above observations are in conformity with Tzafriri *et al*[33] McGinty and Pontrelli,[34] although the embedment of stent has been ignored in the latter study.

Temporal variations of normalised mean drug concentrations are presented in Figure 4. Evidently, the concentrations of free and ECM-bound drug rise from zero to a maximum value and then slowly decay with time; however, the concentration of SR-bound drug steadily increases with time considered. It is worthy to note that the peak of NDC_{SR} occurs later than that of NDC_{FREE} and also more drug is contained within the ECM-bound phase than the other two phases, which are in good agreement with those of Tzafriri *et al*[33] and McGinty and Pontrelli.[34] By choosing $k_{f_{SR}} = 0 = k_{r_{SR}}$, we convert the model considered into the single bound phase model. Here, too, we see that the mean concentration of free drug in case of the single bound phase model is all time higher than that of the double bound phase model, which is due to less conversion of free drug into bound form in the single bound phase model (Fig. 5).

The influence of Damköhler number on the normalised concentrations of free drug, ECM-bound and SR-bound drug in the arterial tissue over a stipulated period of time is displayed in Figure 6a–c respectively. Evidently, the mean concentration of free drug decays rapidly and also the mean concentrations of ECM- and SR-bound drug attain their respective steady state for smaller Damköhler number. Spatial distribution of free, ECM-bound and SR-bound drug concentration can be visualised through Figure 7a–c respectively. These figures clearly establish the impact of time-dependent release kinetics on the transport and retention of

Table 1. Plausible values of involved parameters (dimensional)

Description	Parameter	Value	Reference
Diameter of the strut (cm)	d	0.02	[31,44]
Interstrut distance (cm)	z_d	0.06	[39]
Wall thickness (cm)	δ_w	0.1	[16,45]
Free drug diffusion coefficient ($cm^2 \cdot s^{-1}$)	D_t	10^{-9}	[40,46]
Initial strut drug concentration ($mol \cdot cm^{-3}$)	c_0	10^{-6}	[11]
Association rate constant in ECM-bound phase($(mol \cdot cm^{-3} \cdot s)^{-1}$)	$k_{f_{ECM}}$	2×10^{6}	[33,34]
Dissociation rate constant in ECM-bound phase(s^{-1})	$k_{r_{ECM}}$	5.22×10^{-6}	[33,34]
Local density in ECM binding site($mol \cdot cm^{-3}$)	$\mathfrak{C}_{ECM}^{max}$	3.63×10^{-7}	[33,34]
Association rate constant in SR-bound phase($(mol \cdot cm^{-3} \cdot s^{-1})^{-1}$)	$k_{f_{SR}}$	8×10^{8}	[33,34]
Dissociation rate constant in SR-bound phase(s^{-1})	$k_{r_{SR}}$	1.6×10^{-4}	[33,34]
Local density in SR binding site($mol \cdot cm^{-3}$)	$\mathfrak{C}_{SR}^{max}$	3.3×10^{-9}	[33,34]
Drug release rate(s^{-1})	λ	10^{-5}	[41]

drug in stent-based delivery.

Conclusion and study limitation

In this numerical study, we proposed two-dimensional axi-symmetric models of drug transport eluted from a DES. Following Tzafriri *et al*[33] and McGinty and Pontrelli,[34] we considered a three-phase model, capable of predicting the time-dependent delivery of free drug and its ECM- and SR-binding in arterial tissue with the binding site actions modelled using a nonlinear reversible saturable chemical reaction. The transport of free drug was modelled as an unsteady reaction-diffusion process, while both the bound drugs were modelled as a reaction process only. The simulated results predict the saturation of binding sites that takes place with increasing time and SR-bound drug is totally absorbed at the adventitial boundary. This study also highlights the fact that the concentration of free drug is always higher when the model reduces to two-phase, in which a single bound phase is considered. Another important observation noted is that the concentration of free drug decays rapidly and the earlier saturation of binding sites take place in case of smaller Damköhler

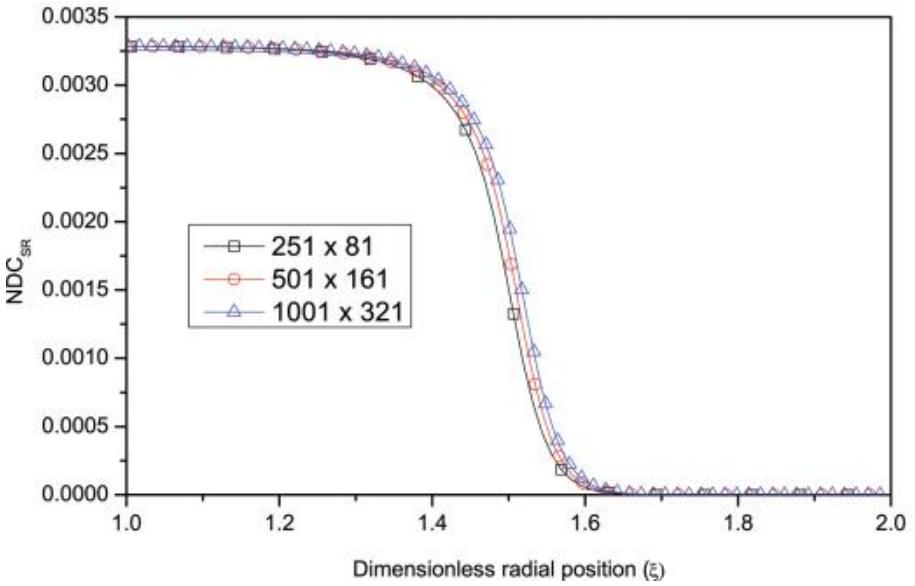

Fig. 2. Transmural variation of normalised bound drug concentration in SR phase (NDC_{SR}) with different grid sizes at z=11 ($Da_{ECM}=8.0\times10^{5}$, $Da_{SR}=32.0\times10^{7}$, $k_{eq_{ECM}}=3.8\times10^{2}$, $k_{eq_{SR}}=5.0\times10^{6}$).

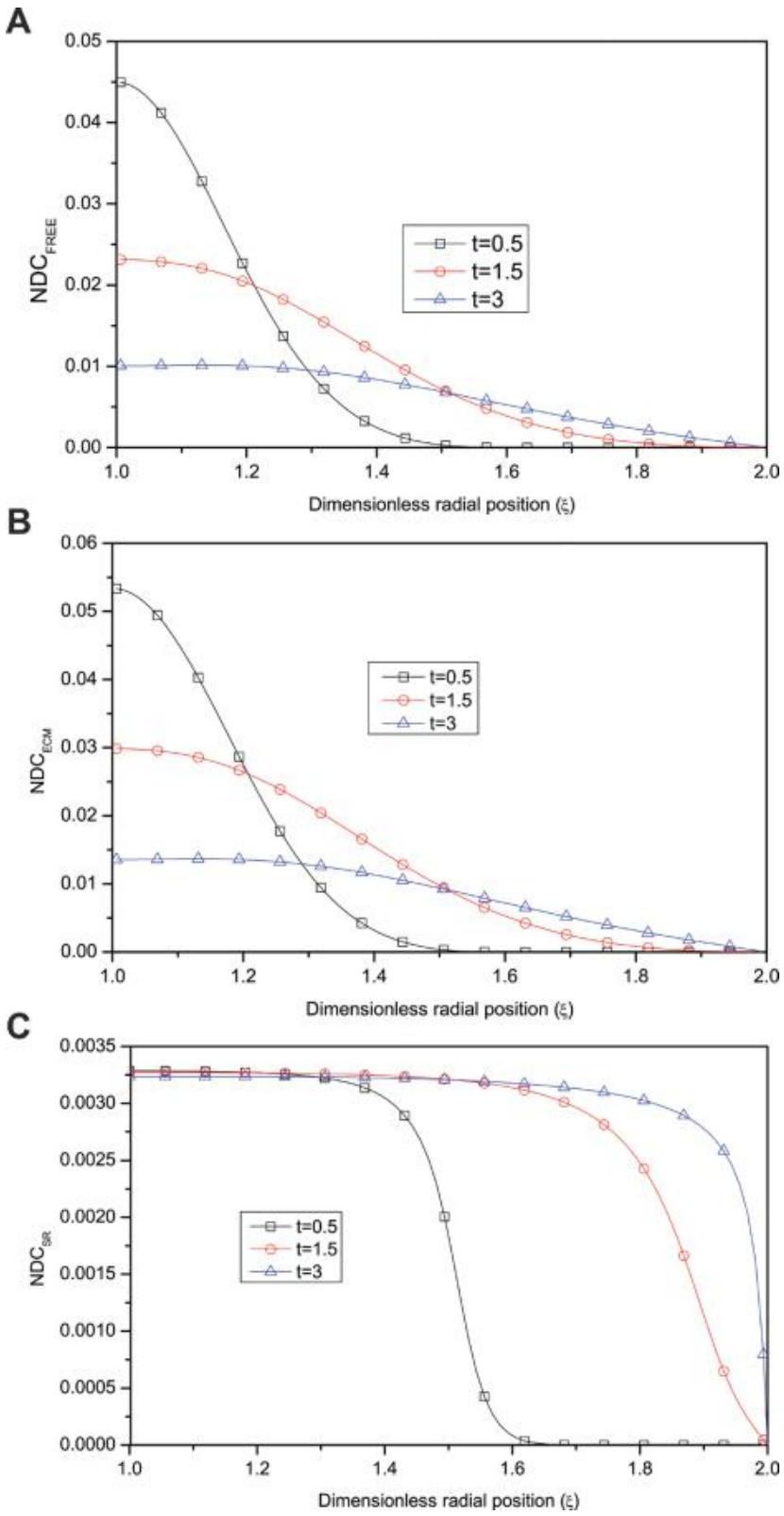

Fig. 3. Transmural variation of normalised drug concentration for different times at z=11. (a) Normalised free drug concentration (NDC_{FREE}), (b) Normalised bound drug concentration in ECM phase (NDC_{ECM}), (c) Normalised bound drug concentration in SR phase (NDC_{SR}) (Da_{ECM}=8.0×10^5, Da_{SR}=32.0×10^7, $k_{eq_{ECM}}$=3.8×10^2, $k_{eq_{SR}}$=5.0×10^6).

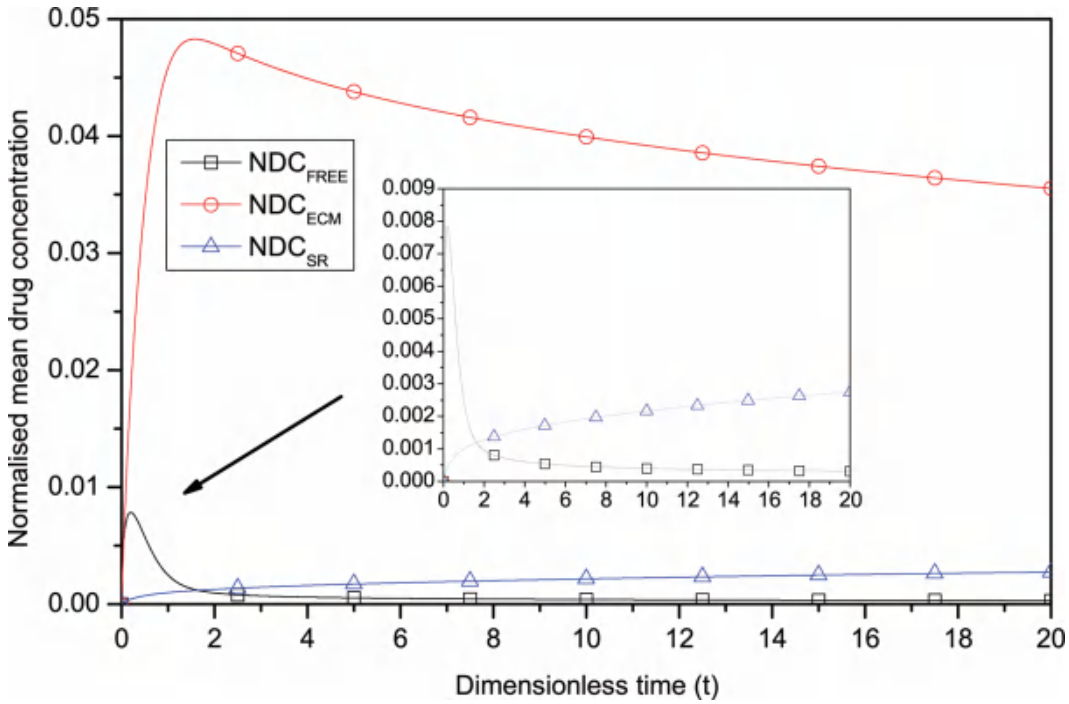

Fig. 4. Temporal variation of normalised mean drug concentration (Da_{ECM}=8.0×10^5, Da_{SR}=32.0×10^7, $k_{eq_{ECM}}$=3.8×10^2, $k_{eq_{SR}}$=5.0×10^6).

number.

As with the case of any computational model study, our study was based on a number of assumptions made regarding selected parameters (Table 1) and the boundary conditions that were assigned due to non-availability of data in the literature. Although not ideal, derivation of all these parameters from human tissues may not be feasible and hence our assumption may be considered as an approximation towards quantifying arterial pharmacokinet-

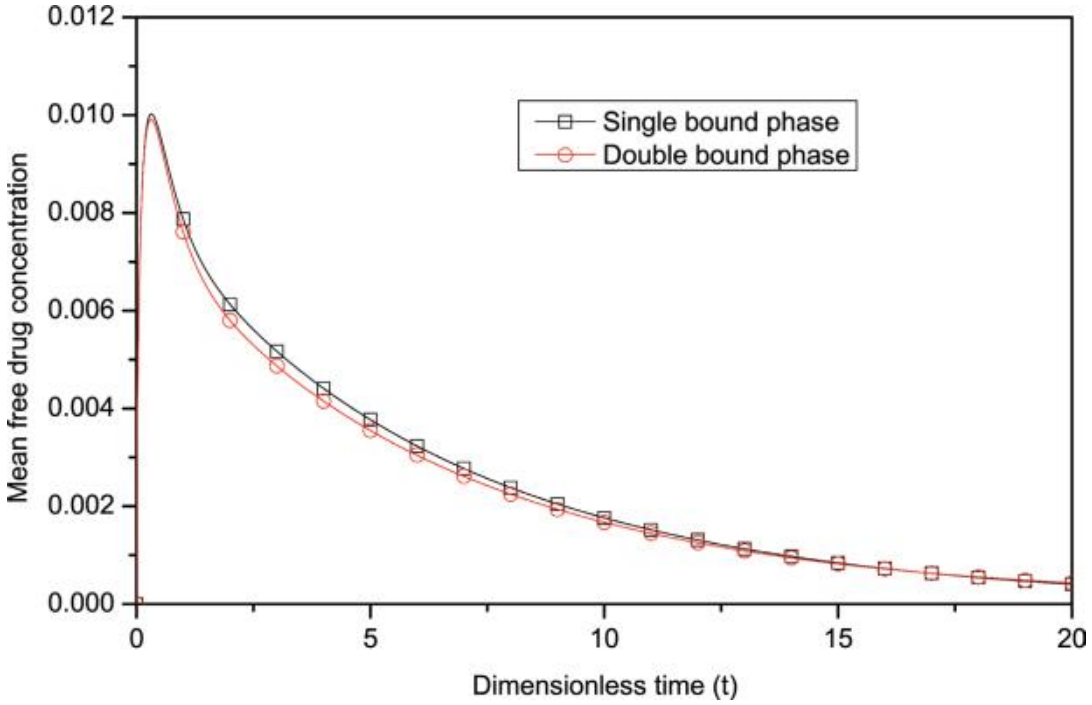

Fig. 5. Temporal variation of normalised mean free drug concentration for different binding phase (Da_{ECM}=8.0×10^5, Da_{SR}=32.0×10^7, $k_{eq_{ECM}}$=3.8×10^2, $k_{eq_{SR}}$=5.0×10^6).

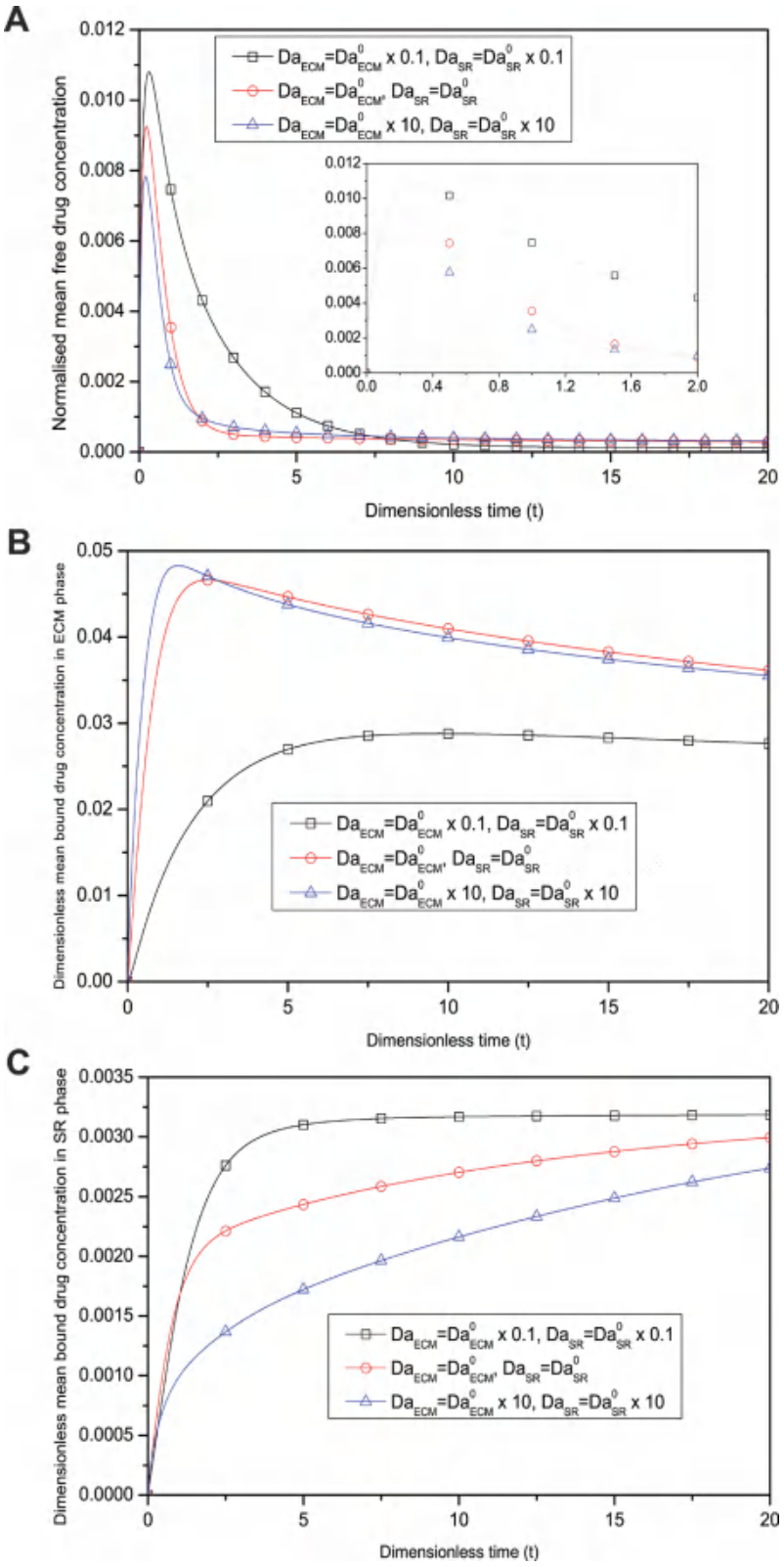

Fig. 6. Temporal variation of normalised mean drug concentration for different Damköhler numbers. (a) Normalised free drug concentration, (b) Normalised bound drug concentration in ECM phase, (c) Normalised bound drug concentration in SR phase (Da_{ECM}=8.0×10^5, Da_{SR}=32.0×10^7, $k_{eq_{ECM}}$=3.8×10^2, $k_{eq_{SR}}$=5.0×10^6).

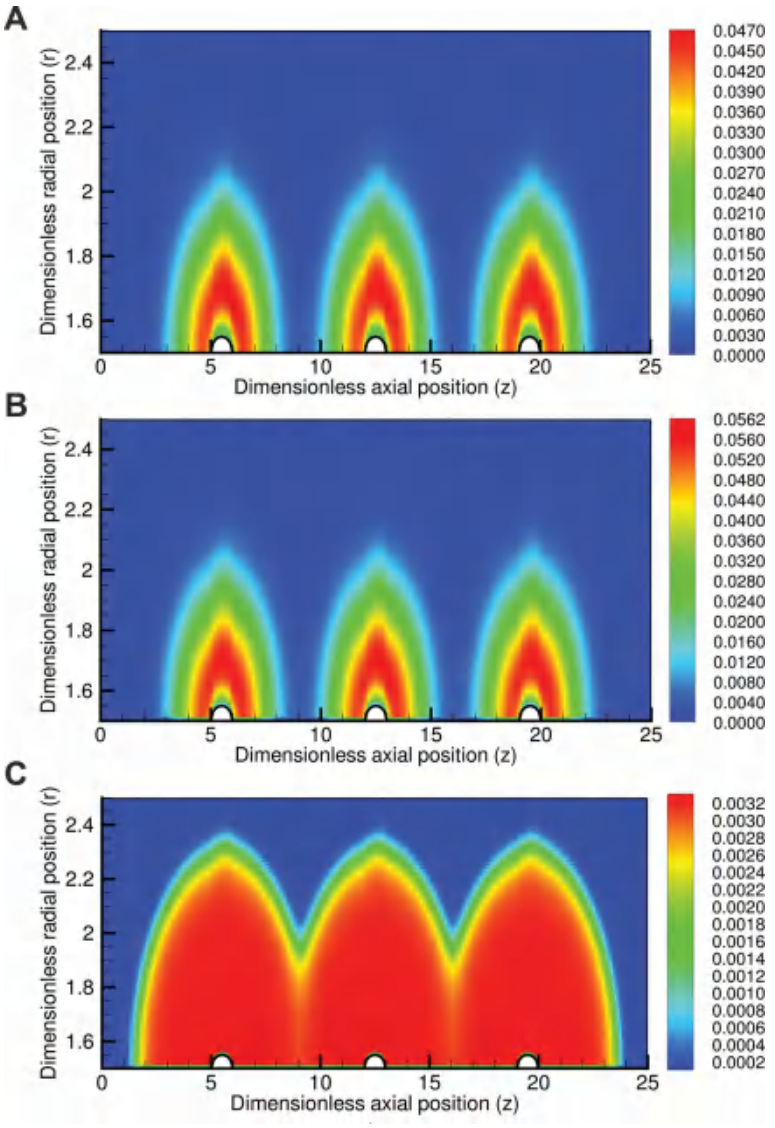

Fig. 7. Visual representation of normalised drug concentration. (a) free drug concentration, (b) bound drug concentration in ECM phase, (c) bound drug concentration in SR phase (Da_{ECM}=8.0×10^5, Da_{SR}=32.0×10^7, $k_{eq_{ECM}}$=3.8×10^2, $k_{eq_{SR}}$=5.0×10^6).

ics in stent-based delivery.

Future direction

With the rapid ascent of stent-based drug delivery in the treatment of vascular disease, many important issues concerning drug delivery and its retention in the arterial tissue need to be addressed. For realistic modelling, the inclusion of luminal flow along with its pulsatility would certainly predict the delivery system one step closer to the real situation. Future directions may also include heterogeneous tissue composition comprising of healthy tissue and regions of fibrous, fibro-fatty, calcified and necrotic core lesions, with varying diffusivities. The effect of porosity and tortuosity of the arterial tissue may not be ruled out for further research.

It is well-established that the presence of stent coating together with its design greatly influence the distribution and retention of drug within the vessel wall but,[33,34] for simplicity, the stent-coating system is not taken into account in the present study, which is in agreement with the numerical evidence proposed in.[8,26,45] Following McGinty *et al*,[47] Zhu and Braatz,[48] the effects of variable porosity, percolation and biodegradability of the stent coating may be distinct topics of future research, and which we intend to include in our further studies.

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Conflict of interest

The authors have no conflict of interests related to this publication.

Author contributions

Designing and performing the research as well as writing the paper (APM and PKM).

Supporting information

Supplementary material for this article is available at https://doi.org/10.14218/JERP.2017.00001.

Data S1. Supplementary Data.

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Original Article

In Vitro Cyto-genotoxicity of Hydroxycitric Acid: A Weight-loss Dietary Supplement

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Abstract

Background/Objectives: The growing issue of clinical obesity has led to increased consumption of weight-loss dietary supplements containing hydroxycitric acid (HCA) derived from the fruit rind of *Garcinia cambogia*, a plant widely distributed in Asia and Africa. It is often consumed in an unregulated manner, beyond the permissible dose, to achieve the target weight-loss. However, its safety/efficacy is controversial and reports on cytotoxicity and genotoxicity are limited and inconsistent. Hence, we aimed to study the putative effects of HCA on genotoxicity in human peripheral blood cells.

Methods: Human lymphocytes and erythrocytes were treated with HCA (0, 10, 20, 40 or 100 µg/mL) for 3 h or 24 h and processed for cytotoxicity and genotoxicity analyses.

Results: Initial phytochemical assessment of HCA revealed the presence of high flavonoid content. Subsequent multi-endpoint cyto-genotoxicity studies in human lymphocytes displayed low cytotoxicity but significant genotoxicity at higher concentrations of 40 and 100 µg/mL; these concentrations are approximately equivalent to and double the maximum permissible dose (~2800 mg/day), respectively. Flow cytometric estimation of reactive oxygen species (ROS), mitochondrial membrane potential (ΔΨm) and mode of cell death revealed significant ROS generation at the higher concentrations, but no effect on ΔΨm and apoptosis/necrosis. Insignificant hemolysis was observed in erythrocytes.

Conclusions: High flavonoid content of HCA potentially imparts pro-oxidant property, facilitating DNA damage at high concentrations. However, such genotoxicity does not lead to cell death. Therefore, HCA can be recommended for safe consumption within the permissible dose limit.

Introduction

Obesity–a rising public health concern in all industrialized nations of the world–is often designated as an epidemic, affecting about 600 million adults worldwide and with an annual death toll of 300,000 in South-East Asia.[1] As a result, consumption of alternative anti-obesity medications has gained popularity. Among them, weight-loss dietary supplements containing hydroxycitric acid (HCA), derived from the dried fruit rind of *Garcinia* sp. (16–26% HCA), include a major fraction, with a permissible dose of 15–47 mg/kg/day (900~2800 mg/day for a person weighing 60 kg).[2] Nevertheless, clinically obese patients have the propensity to consume unregulated doses in order to achieve their target weight. The fruit of *Garcinia* sp. is used in traditional Chinese medicine for its anti-oxidant properties, which can be partly attributed to the presence of HCA.[3,4] It performs competitive inhibition of ATP citrate lyase, an extra- mitochondrial enzyme which catalyzes the formation of the primary building blocks of fatty acid and cholesterol biosynthesis, oxaloacetate and acetyl coenzyme A from citrate coenzyme A.[5]

Cyto-genotoxicity studies on HCA are limited and their results are often conflicting, since different investigators have used different production processes, concentrations, *Garcinia* sp. and sampling points; the contradictions may also be due to the structural

Keywords: Comet assay; DNA diffusion; ROS; Mitochondrial membrane potential; Apoptosis; Hydroxycitric acid.

Abbreviations: HCA, hydroxycitric acid; ROS, reactive oxygen species; ΔΨm, mitochondrial membrane potential; ATP, adenosine triphosphate; TB test, trypan blue dye exclusion test; MTT, 3-(4,5-dimethythiazol- 2-yl)-2,5-diphenyl tetrazolium bromide; TPC, total phenolic content; TFC, total flavonoid content; OECD, Organisation for economic co-operation and development; MMS, methylmethanesulfonate; PI, propidium iodide; DCFH-DA, 2′, 7′dichlorofluorescein diacetate; FITC, fluorescein isothiocyanate.

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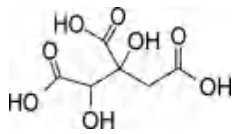

Fig. 1. Chemical structure of hydroxycitric acid.

instability of HCA, as it alters from open chain to lactone form, which is stabilized by counter ions such as potassium or calcium.[5] *In vivo* genotoxicity studies have revealed the induction of chromosome aberration (CA), sister chromatid exchange (SCE) and micronuclei (MN).[6,7] Paradoxically, recent *in vitro* studies did not reveal genotoxicity but showed cytotoxicity.[8,9]

Thus, in view of the recent incongruent scientific findings and the rising demand for HCA, the present study was undertaken using multiple cytotoxic (trypan blue dye exclusion (TB) test and MTT assay), genotoxic (comet, DNA diffusion assays) endpoints for the first time, with emphasis on their mechanisms by detection of reactive oxygen species (ROS) generation (to reflect genotoxicity), mitochondrial membrane potential (signified by ΔΨm) and apoptosis/necrosis (to reflect cytotoxicity) in human lymphocytes. The hemolytic effect of HCA in erythrocytes was also assessed. The assays for total polyphenolic content (TPC) and total flavonoid content (TFC) were employed for an initial phytochemical analysis. HCA showed high TFC, absence of cytotoxicity and hemolysis, but induced genotoxicity at concentrations higher than that recommended daily.

Methods

Test substance

Calcium salt of HCA (extracted from the fruit rind of *Garcinia cambogia*) was procured from Arjuna Natural Extracts Limited (Kerala, India) (Product code: GCC-073; CAS No. 90045-23-1). Figure 1 represents the chemical structure of HCA. The percentage of HCA was 50.9% (by high-performance liquid chromatography), with calcium content of 13–19% and salt content of <2.5%, as provided by the manufacturer. The lactone and citric acid contents were 1.2% and 3.1%, respectively.[10] This compound containing ~50% HCA was used for toxicity studies without further purification, as it is consumed in its crude form.

Phytochemical analysis

Ethanolic extracts of HCA (5, 7.5 and 10 mg/mL) were used for phytochemical screening.TPC was estimated according to the method of Singleton *et al*.[11] using gallic acid as the standard. For TFC, the method of Chang *et al*.[12] was used with the minor modifications described by Nag *et al*.[13] with quercetin as the standard. TPC was measured spectrophotometrically (Beckman Coulter, CA, USA) at 765 nm and expressed in terms of mg gallic acid equivalent per g HCA extract (mg GAE/g). TFC was estimated at 420 nm (Beckman Coulter) and calculated as mg quercetin equivalent per g HCA extract (mg QUE/g). All experiments were performed in triplicate.

Test systems and treatment

Human blood was drawn by venipuncture from 3 healthy male adult volunteers (non-smokers, non-alcoholics and not consuming any medication), with their consent and following the Organisation for Economic Co-operation and Development (OECD) guidelines.[14] Lymphocytes from each donor were isolated separately following the method of Boyum,[15] using Histopaque density gradient followed by resuspension in RPMI-1640 media at a concentration of 2×10^6 cells/mL. Cell populations with >98% viability, as determined by TB test, were used.[16] Erythrocytes were separated as previously described by Ghosh *et al*.[17] and diluted in phosphate-buffered saline (PBS) to a concentration of 2×10^6 cells/mL.

Selection of the concentration of HCA for study was based on preliminary cytotoxicity tests, previously published literature and recommended daily dosage.[2] Cytotoxicity analysis was performed using MTT assay in concentrations ranging from 0–200 µg/mL HCA, where the cut-off point was deemed to be 70% cell viability as reported by Henderson *et al*.[18] The freshly isolated lymphocytes and erythrocytes were incubated with 0, 10, 20, 40 or 100 µg/mL HCA in RPMI-1640 media, along with positive control compounds (100 µM H_2O_2 for TB, MTT, ΔΨm, apoptosis/necrosis, ROS assays; 100 µM methylmethanesulfonate (MMS) for comet and DNA diffusion assays; and 1 % TritonX-100 for hemolysis test(data not shown)) at a density of 1×10^6 cells/mL per concentration, with exposure for 3 h or 24 h at 37 °C.

All experiments were approved with ethical clearance by the Research Ethics Committee of University of Calcutta, India. The cells were treated with each concentration of HCA separately for each donor without pooling. To achieve acceptable results, all experiments were repeated thrice from blood collected from each donor and each of the treatment concentrations were analyzed in triplicate.

Cytotoxicity analyses

TB test and MTT assay were performed according to the methods of Tennant and Mosmann, respectively, with modifications.[16,19,20] In the TB test, cell viability was scored using a Neubauer hemocytometer under a light microscope (Leica, Wetzlar, Germany). For MTT, the optical density (OD) values were read (iMark™ Microplate Absorbance Reader; Bio-Rad, CA, USA) at 570 nm, with 630 nm as reference wavelength.

For ΔΨm, the cells were washed in PBS, stained with rhodamine 123 (10 µM in PBS) and incubated for 15 min at 37 °C in the dark.[17,21] Apoptotic and necrotic cell death was quantified using the annexin V-FITC/PI staining method by an apoptosis/necrosis detection kit (BD Pharmingen, CA, USA).[20,22] The cells were washed with PBS, resuspended in calcium binding buffer (100 µL), stained with annexin V-FITC (5 µL) and propidium iodide (PI) (5 µL; 1 µg/mL) for 15 min in the dark. Approximately 10,000 events were analyzed for both ΔΨm and apoptosis/necrosis by flow cytometry (BD FACS Verse™; Becton Dickinson, NJ, USA). Data analysis was carried out using the BD FAC Suite software, version 1.0.5.3841 (Becton Dickinson). The percentages of viable (PI^-, annexin V^-), early apoptotic (annexin V^+, PI^-), late apoptotic (annexin V^+, PI^+) and necrotic (annexinV^-, PI^+) cells were calculated. The results of ΔΨm were expressed as fold-change over control.

Genotoxicity analysis

Comet assay was performed following the method of Tice *et al*.[23], with modifications.[20] Slides were prepared in triplicate per concentration;image procurement (Leica) and data analysis

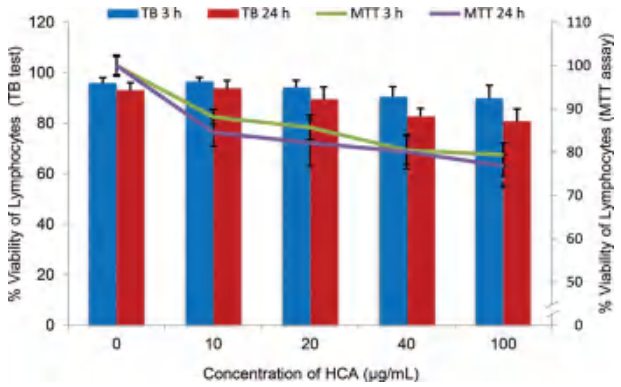

Fig. 2. Cytotoxicity evaluation of HCA at 0, 10, 20, 40 and 100 µg/mL in human lymphocytes after 3 h and 24 h by trypan blue dye exclusion test and MTT assay. **p*< 0.05 *vs.* control, one-way ANOVA, *n*=3. Abbreviations: HCA, hydroxycitric acid; TB test, trypan blue dye exclusion test; MTT, 3-(4,5-dimethythiazol- 2-yl)-2,5-diphenyl tetrazolium bromide.

were carried out using Komet 5.5 software (Kinetic Imaging, Nottingham, UK). Among the comet parameters, we report the medians of the percent tail DNA from 300 (100×3) nuclei/concentration. The number of hedgehog structures were counted manually (300 nuclei/concentration; 100/slide) and expressed as percent.[24]

DNA diffusion assay was performed according to the method of Gichner *et al*.[25], with modifications.[20] Slide processing was the same as for the comet assay, but without subjectionto electrophoresis. The slides were placed in spermine solution (1 mg/mL in 50% ethanol) for 30 min after lysing. Staining, visualization and data analysis was the same as in the comet assay. The percentage of nuclear area of 300 (100×3) nuclei per concentration was used to express nuclear DNA diffusion and the percentage of diffused nuclei was calculated manually.

The treated lymphocytes were incubated in 2′, 7′ dichlorofluorescein diacetate (DCFH-DA) (25 µM in PBS) for 30 min at 37 °C in the dark for ROS analysis.[17,26] About 10,000 events were analyzed by flow cytometer using the same instrument and data analysis software as for the study of ΔΨm and apoptosis/necrosis. Data are expressed as fold-change over control.

Effect on erythrocytes by hemolysis test

The treated erythrocyte suspensions were analyzed for hemolysis according to the method of Katsu *et al*.[27], with modifications.[17] The degree of hemolysis was estimated by measuring the absorbance of the supernatant at 540 nm (Beckman Coulter). Results are expressed as:

$$\% \text{ hemolysis} = \left[\frac{a-b}{c-b}\right] \times 100$$

Where a = Absorbance$_{\text{Sample}}$, b = Absorbance$_{\text{Negative control}}$, and c = Absorbance$_{\text{Positive control}}$

Statistical analysis

One-way ANOVA (Sigma Plot 12.0, Systat Software Inc., CA, USA) was performed and the level of statistical significance was established at $p < 0.05$. Multiple comparisons were carried out using Duncan's multiple range tests. All data are presented as mean ± SEM of three replicates.

Results

Phytochemical analysis of HCA

TPC and TFC of HCA were assessed for phytochemical analysis. TPC was estimated to be 0.105 ± 0.022 mg GAE/g, as determined by reference to the standard curve of gallic acid (y = 0.088x + 0.132, $R^2 = 0.99$). A high TFC of 8.94 ± 0.082 mg QUE/g was detected, by reference to the standard curve of quercetin (y = 0.037x + 0.0017, $R^2 = 0.99$).

Effect of HCA on cell viability, mitochondrial function and mode of cell death in human lymphocytes

Figure 2 demonstrates the absence of significant induction of cytotoxicity, as evaluated by TB test and MTT assay. No significant decline in cell viability was observed by TB test at either time interval ($p < 0.05$), demonstrating lack of effect on cell membrane permeability. Exposure to the highest concentration of HCA (100 µg/mL) led to 89.88% and 80.83% cell viability at 3 h and 24 h, respectively. The percent cell viability with the positive control H_2O_2 (100 µM) was 81.7% and 73.2% at 3 h and 24 h, respectively. MTT assay displayed no significant decline in cell viability, indicating no effect on the mitochondrial dehydrogenase activity after 3 h and 24 h, with the exception of a significant decline (76.79%) at the highest concentration of 100 µg/mL at 24 h (Fig. 2). IC_{50} values of HCA ranged from 29.330 ± 0.072 mg/mL at 3 h to 27.695 ± 0.069 mg/mL at 24 h exposure. The positive control H_2O_2 (100 µM) revealed percent cell viability of 69.41% and 56.69 % at 3 h and 24 h, respectively.

The cationic fluorescent probe rhodamine 123 revealed insignificant depolarization of the mitochondrial membranes (ΔΨm) upon HCA treatment at both time points (Fig. 3). The least decline in the intensity of rhodamine 123 fluorescence quantified at 24 h was ~21% less than control in 100 µg/mL HCA-treated cells.

The mode of cell death was assessed by annexin V-FITC/PI double-staining. There was insignificant rise in early or late apoptotic cells and negligible occurrence of necrotic cells at all treatment concentrations (Fig. 4). These results mirror the MTT assay data. Thus, the unaffected mitochondrial dehydrogenase activity, unaltered mitochondrial metabolic activity and the retention of intact mitochondrial membranes are correlated with the absence of apoptosis. The meager frequency of necrotic cells can be corroborated with the TB test data showing lack of cell membrane damage.

Evaluation of genotoxicity and oxidative stress in human lymphocytes

HCA-induced DNA damage (tail DNA percent) was statistically significant at concentrations of 40 and 100 µg/mL, at both time points,as observed by comet assay. The percent tail DNA values were approximately 6- and 7-fold higher than the respective controls at 100 µg/mL after 3 h and 24 h, respectively (Fig. 5a). These concentrations are almost identical to and approximately double the maximum permitted dose (*i.e.* 900–2800 mg/day or 15–47 mg/kg/day, respectively).[2] The percent increase in hedgehogs followed a similar pattern as the comet results ($p < 0.05$). The positive control MMS (100 µM) showed 62.9% and 76.42% tail DNA and led

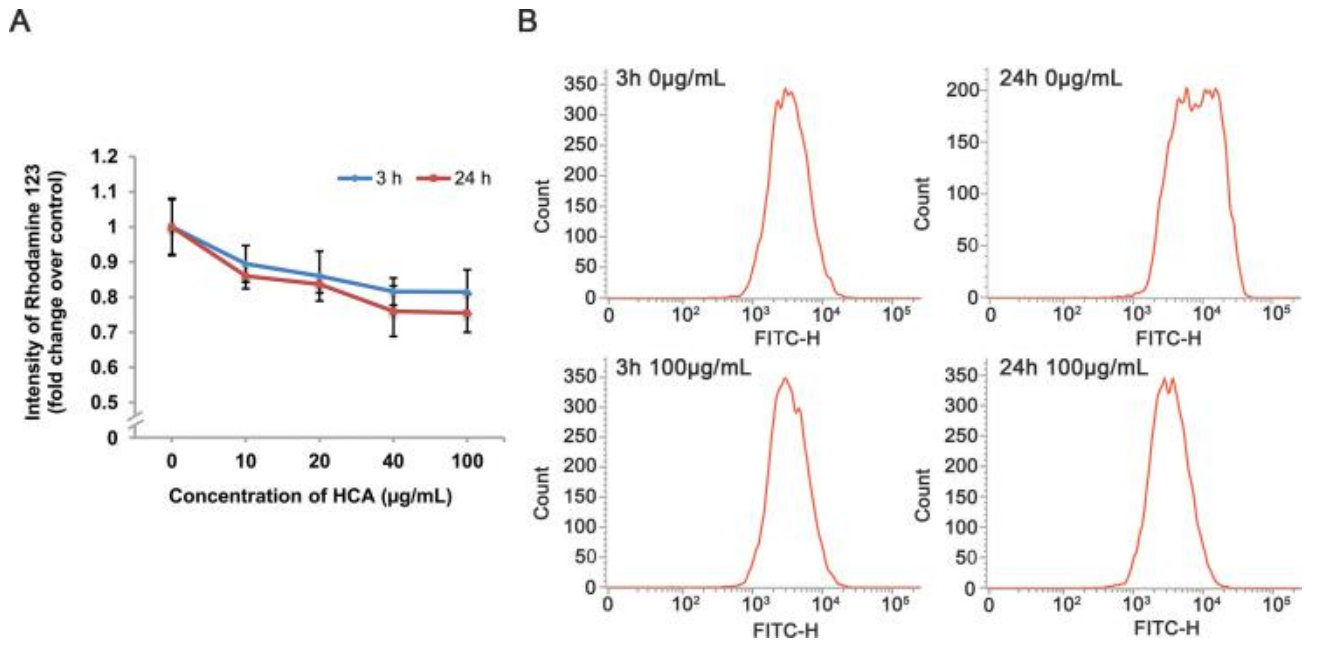

Fig. 3. Flow cytometric estimation of mitochondrial membrane potential by rhodamine 123 staining in human lymphocytes treated with various concentrations of HCA for 3 h and 24 h. (a) Intensity of rhodamine 123 fluorescence as fold-change over control. (b) Representative flow cytometry histograms of control and the highest treatment concentration of HCA (100 µg/mL) at 3 h and 24 h time intervals. *p< 0.05 *vs.* control, one-way ANOVA, n=3. Abbreviations: HCA, hydroxycitric acid; FITC, fluorescein isothiocyanate.

to the formation of 37.2% and 52.4% hedgehogs, after 3 h and 24 h, respectively. DNA diffusion under alkaline/neutral conditions is a widely-accepted biomarker of DNA strand breaks that lead to apoptotic/necrotic cell death.[24] Figure 5b shows the minor rise in the percent nuclear area and frequency of diffused nuclei upon HCA treatment, which is indicative of low cytotoxicity. The highest treatment concentration of HCA (100 µg/mL) induced significant increase in the percent nuclear area at 3 h and 24 h compared to the respective controls. MMS (100 µM) induced substantially high increase in the percent nuclear area and the percent diffused nuclei at 3 h and 24 h.

As a mechanism of DNA damage, oxidative stress was evaluated using the hydrophobic non-fluorescent dye DCFH-DA,which infiltrates cells rapidly and is hydrolyzed by intracellular esterases to produce DCFH. The oxidation of DCFH by intracellular ROS to its fluorescent 2-electron product 2′, 7′dichlorofluorescein (DCF) was quantified by flow cytometry. Significant increase in ROS production was found at concentrations of 40 µg/mL and above at both the 3 h and 24 h times compared to the respective controls (Fig. 6). The results were approximately 2- and 3-fold higher for 100 µg/mL of HCA exposure than that of control at 3 h and 24 h, respectively.

Taken together, the results suggest that HCA-induced genotoxicity may not lead to apoptotic/necrotic cell death. Such DNA damage can be attributed to oxidative stress, which is independent of mitochondrial ROS generation, as reflected by negligible decline in ΔΨm.

Estimation of hemolytic potential

Toxicity studies on lymphocytes were succeeded by an analysis of the hemolytic potential of erythrocytes. As shown in Figure 7, there was no significant rise in percent hemolysis at either 3 h or 24 h compared to control.

Discussion

The rising problem of clinical obesity has led to the increased usage of HCA derived from *Garcinia* sp. as the chief component of weight-loss dietary supplements, thereby necessitating its thorough toxicological evaluation. Reports on its safety and efficacy are conflicting, and the published *in vitro* studies using cyto-genotoxic endpoints are limited. Therefore, the present investigation was carried out using human lymphocytes and erythrocytes to provide information on (a) cytotoxicity and mitochondrial function, (b) genotoxicity and oxidative stress, and (c) hemolysis induced by HCA.

Phytochemical screening of HCA in terms of TPC and TFC was undertaken to understand the correlation between its chemical constituents and their biological effects *in vitro*. The TFC (8.94 ± 0.082 mg QUE/g) of HCA was higher than the TPC (0.105 ± 0.022 mg GAE/g). Present literature is replete with diverse values of TPC and TFC which vary with extraction solvents (ethanol, methanol, water) and *Garcinia* species.[3,4] High flavonoid content of *G. cambogia* fruit extracts (ethanolic/methanolic and water) ranges between 0.137–30 mg QUE/g.[3,4] Our result of high TFC for pure Ca salt of HCA derived from *G. cambogia* fruit rind is within the range of these available values for crude *G. cambogia* extracts. Such high TFC of HCA is also validated by previous studies and is reported to cause hypolipidemic activity.[28]

In our study, HCA was non-cytotoxic to human lymphocytes, as demonstrated by TB test and MTT assay,and showed negligible mitochondrial dysfunction at the tested concentrations. Findings reported from elsewhere have indicated that *G. atroviridis* acid ester derivatives containing HCA were non-cytotoxic in CEMSS (human T-lymphoblastic leukemia) cells and Raji (human B-lymphoblastoid) cells.[29] On the other hand, exposure of 3T3 fibroblast

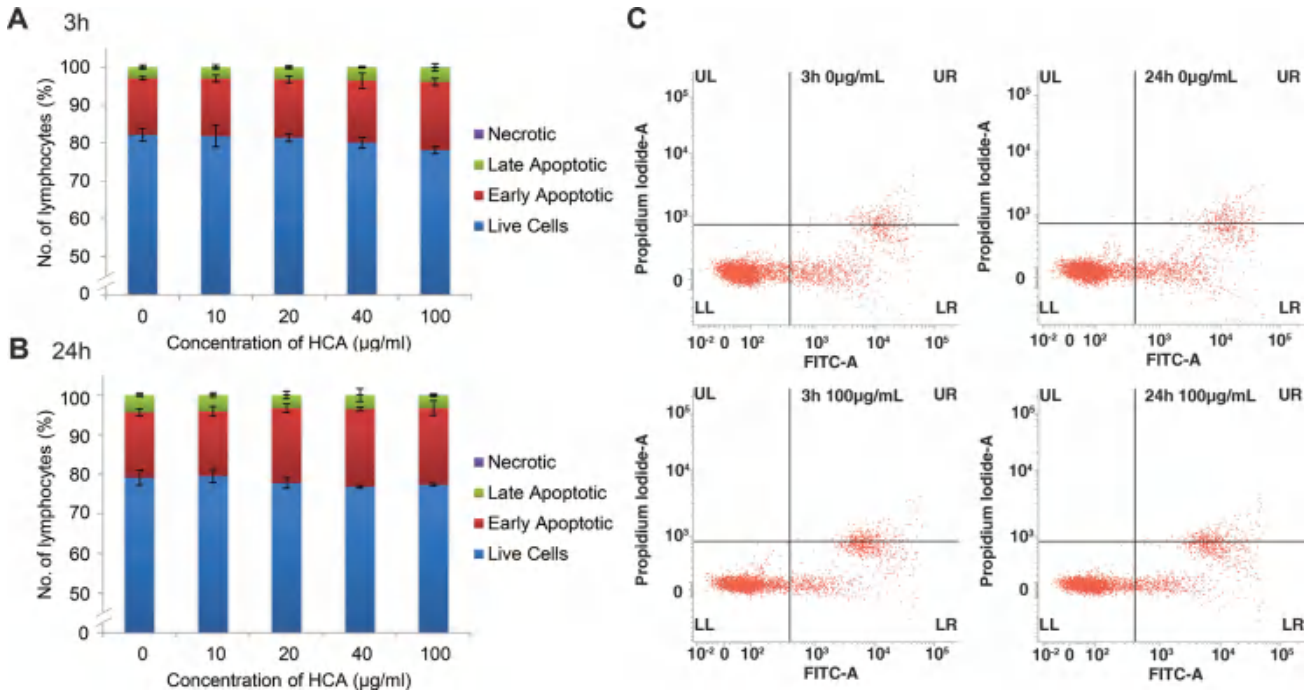

Fig. 4. Mode of cell death assessed by annexin V/FITC-PI staining in human lymphocytes treated with 0, 10, 20, 40 and 100 µg/mL HCA after 3 h and 24 h. Frequency of live, early apoptotic, late apoptotic and necrotic lymphocytes after (a) 3 h and (b) 24 h. (c) Representative flow cytometry dot plots showing the distribution of events according to the intensity of FITC and propidium iodide scattering among the quadrants segregated as LL (live cells), LR (early apoptotic cells), UR (late apoptotic cells) and UL (necrotic cells) at control and the highest HCA concentration (100 µg/mL). *p< 0.05 *vs.* control, one-way ANOVA, n=3. Abbreviations: HCA, hydroxycitric acid; FITC, fluorescein isothiocyanate.

cells to *G. indica* crude extracts diluted in dimethyl sulfoxide at a concentration of 240 µg/mLled to nearly 80% decline in cell viability after 6 days.[9] Such divergent results may be due to differences in treatment duration, test system, type of extracts and dilution solvent. Therefore, we considered it prudent to evaluate the mechanisms of induced cytotoxicity with regard to mitochondrial membrane potential and mode of cell death by flow cytometry.

Alteration in mitochondrial membrane potential (as signified by ΔΨm) as a result of mitochondrial dysfunction and subsequent apoptosis is a key mechanism of cytotoxicity.[21] The effect of HCA on mitochondrial function was negligible, as observed by the uptake of the positively-charged fluorescent dye rhodamine-123 by mitochondrial membranes of the treated lymphocytes. These results are congruent with the findings of MTT assay and the mode of cell death analyzed by annexin V-FITC/PI double-staining. Hence, we provide the first report of Ca salt of HCA derived from *G. cambogia* being non-cytotoxic to human lymphocytes.

HCA-induced genotoxicity was significant at high concentrations (40 and 100 µg/mL) for both time intervals examined. Importantly, these concentrations are nearly equivalent to and higher than the permissible dose (*i.e.* 2800 mg/day), respectively.[2] The rise in hedgehog frequency and nuclear area validated the increase in percent tail DNA by comet assay, although such DNA strand breaks did not culminate in apoptosis/necrosis. In this regard, it can be assumed that repair enzymes may be involved to prevent the onset of cytotoxicity as a result of a continuous process of DNA damage.

Hedgehogs are comet structures with pin-like heads and nearly all DNA concentrated in the tail. Recent reports have affirmed the occurrence of hedgehogs as an upper end of a continuous process of DNA damage and not diagnostic of apoptosis/cytotoxicity, as previously believed.[24] Hence, DNA diffusion assay was used in this study to distinguish apoptotic nuclei having dense central nuclear DNA with a hazy and lighter halo-like outer zone from necrotic nuclei with well-defined outer boundary and relatively distinct appearance.[25] Among the diffused nuclei, we observed apoptotic nuclei rather than necrotic nuclei. This is in agreement with the negative finding of necrotic cells by TB test and the annexin V-FITC/PI double-staining data showing minor but insignificant rise in early apoptotic cells and negligible necrotic cell populations.

Our group recently affirmed the DNA damaging potential of *G. indica* fruit extracts in mice by the cytogenetic endpoints of SCE and CA.[6] Lee and Lee,[7] reported significant induction of MN at higher concentrations of HCA-SX (Ca/K salt of 60 % HCA) in mice.[7] In particular, chromosome aberration tests performed in Chinese hamster ovary cells exposed to HCA-SX revealed absence of chromosome aberrations.[7] In another study, comet assay of human blood treated for 4 h with crude *G. cambogia* extracts (at 125 and 250 µg/mL concentrations) showed a steady but statistically insignificant rise in DNA damage.[8] Furthermore, *in vivo* cytogenetic studies and *in vitro* bacterial mutagenicity analyses were negative for citric acid and its sodium and tripotassium salts present in HCA formulations, proving HCA to be the chief cause of genotoxic responses.[30]

These contrasting genotoxicity findings in the literature lead us to hypothesize that the DNA strand breaks induced by HCA at higher concentrations may not always progress to form chromosome aberrations *in vitro*, but may induce MN, chromosome aberrations and SCE *in vivo*. The likelihood of such DNA damages being repaired over time and not terminating into any genetic hazard cannot be ruled out. Similar flavonoid-rich phytochemicals such as curcumin from *Curcuma amada* rhizome and *Punica granatum* L. (pomegranate) whole fruit extracts have been reported to be non-

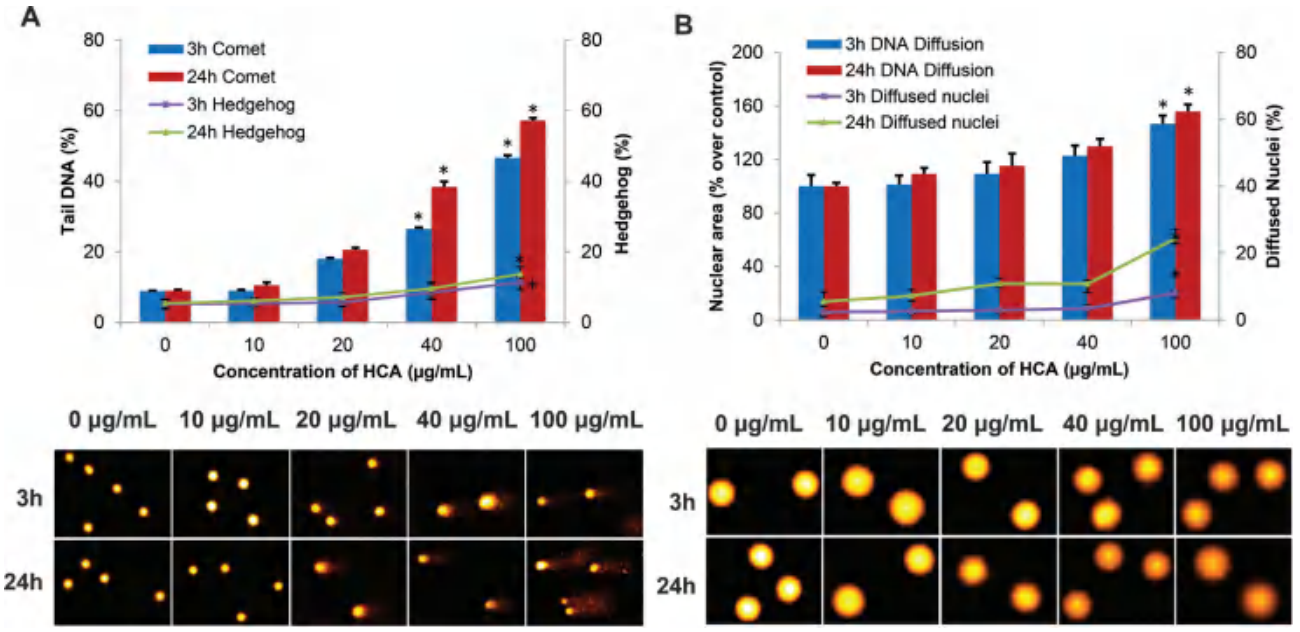

Fig. 5. Genotoxicity analysis in human lymphocytes treated with 0, 10, 20, 40 or 100 µg/mL HCA after 3 h and 24 h. (a) Comet assay (percent tail DNA and percent hedgehogs). (b) DNA diffusion assay (percent nuclear area and percent diffused nuclei). *p< 0.05 *vs.* control, by one-way ANOVA, n = 3. Abbreviations: HCA, hydroxycitric acid.

cytotoxic but genotoxic at higher concentrations both *in vitro* and *in vivo*.[31,32]

Oxidative stress is a known mechanism of DNA damage.[17,20] Therefore, estimation of ROS in human lymphocytes was carried out in this study to investigate the mechanism of the genotoxic responses observed in comet assay. A significant increase in ROS was noted at the similar higher concentrations that showed genotoxic response. In this regard, studies on the potential toxicity of plant extracts rich in flavonoids by others have affirmed their prooxidant activities and possible genotoxic responses at higher concentrations.[33] Thus, high TFC of HCA can be a probable cause of ROS generation at higher concentrations.

Minimal decline in ΔΨm and subsequent low cytotoxicity implies the ability of intracellular antioxidant systems to regulate ROS levels lower than the minimum threshold limits necessary for cell injury/death. This also leads to the assumption of possible in-

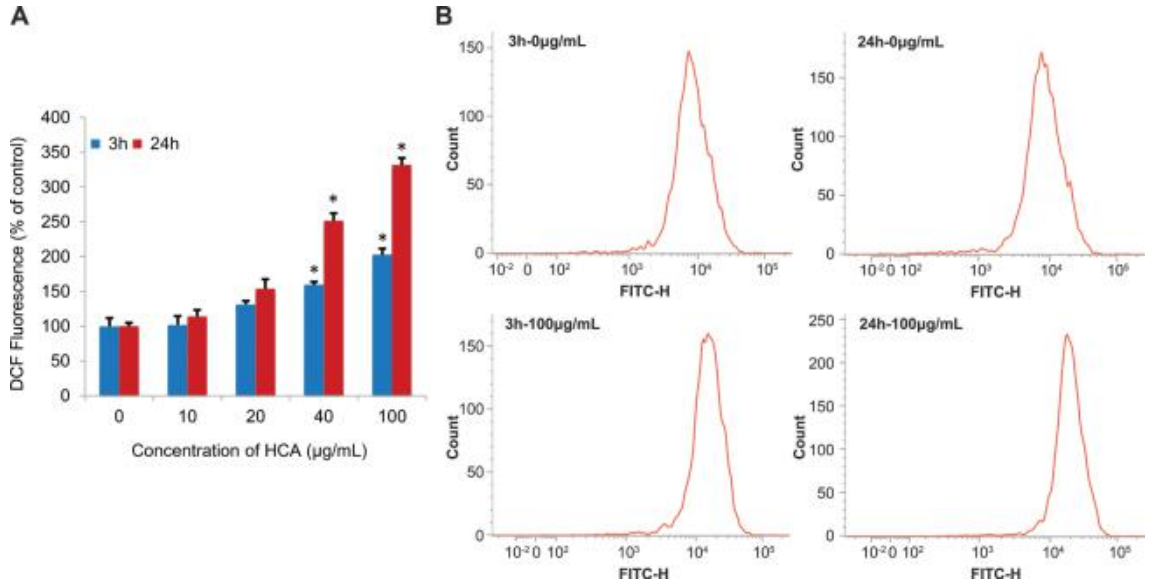

Fig. 6. Dose-dependent induction of ROS generation in human lymphocytes treated with 0, 10, 20, 40 or 100 µg/mL HCA for 3 h and 24 h. (a) Fold-change of 2′, 7′dichlorofluorescein fluorescence over control. (b) Representative flow cytometry histograms of the treated lymphocytes at control and the highest HCA concentration (100 µg/mL) after 3 h and 24 h. *p< 0.05 *vs.* control, by one-way ANOVA, n = 3. Abbreviations: HCA, hydroxycitric acid; ROS, reactive oxygen species; FITC, fluorescein isothiocyanate.

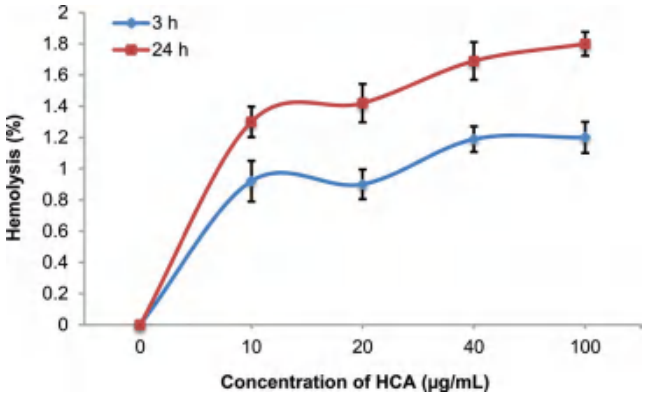

Fig. 7. Hemolytic effect of HCA on human erythrocytes. Data are presented as percent hemolysis. Abbreviations: HCA, hydroxycitric acid.

volvement of other known cellular organelles such as endosomes, lysosomes and Golgi apparatus, instead of mitochondria, in the generation of ROS.[34,35] Therefore, DNA damage incited by HCA at high concentrations is ROS mediated and may be, putatively, repaired over time with the activation of cellular ROS quenching mechanisms since such genotoxic responses did not culminate in cell death. Further studies involving prolonged exposure are required to affirm such a premise.

In agreement with our findings in lymphocytes, HCA did not display any hemolytic potential in erythrocytes in our study either. A previous study on *G. cambogia* fruit extracts also revealed absence of hemolytic activity.[8] Moreover, the same extracts also showed erythropoetic activity in Wistar rats.[36]

Hence, additional studies are needed to assess the DNA damage and repair pathways associated with HCA exposure at high concentrations. Moreover, our results ascertain the safe consumption of HCA within the acceptable dose limit (~2800 mg/day).

Conclusion and future perspectives

The findings of this study provide support for the absence of hemolysis in erythrocytes and cytotoxicity of HCA in human lymphocytes. The genotoxic potential of HCA as a result of oxidative stress was noted at tested concentrations that were high and beyond the permissible limit. Its high flavonoid content may impart pro-oxidant properties, resulting in ROS generation and leading to DNA damage. However, the possibilities of ROS quenching by the cellular antioxidant system facilitating DNA repair over time cannot be ruled out. Since our study was composed of only *in vitro* experiments, further studies are required to understand the DNA damage and repair mechanisms incited by HCA at different time points, both *in vitro* and *in vivo*, for its safer and more efficient long-term usage. Hence, consumption of HCA at low concentrations can be recommended for weight loss in obese and overweight individuals,with the aim of motivating them to embrace a healthier diet with regular exercise.

Acknowledgments

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Conflict of interest

The authors declare no conflicts of interest related to this publication.

Author contributions

Research design (AM), performing experiments (IG), data analysis (IG, AM), manuscript preparation (AM, IG), revision and proofreading (IG, AM).

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Review Article

Pathophysiology, Pharmacology and Treatment of Acute Intermittent Porphyria: A Patient Case Description and Recommendations from the Current Literature

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Abstract

Acute intermittent porphyria (AIP) is a rare and potentially life-threatening metabolic disorder. It is characterized by an autosomal dominant enzymatic deficiency in porphobilinogen deaminase, which is a critical enzyme in the heme biosynthesis pathway. This deficiency leads to an overproduction of porphyrin precursors that can lead to acute attacks that can be severe and affect overall quality of life. These attacks can be precipitated by factors such as medications, nutritional changes, infection and environmental exposures. Liver transplantation is a potential cure for patients who have evidence of end-stage liver disease or are experience multiple life-threatening attacks. This article presents the case of a patient with AIP, who was successfully treated with liver transplantation. The article also provides a review of the epidemiology/pathophysiology of AIP, and its diagnosis and precipitating factors leading to exacerbation of symptoms, as well as its treatment options, with an emphasis on use of liver transplantation to achieve cure.

Case presentation

A 30-year-old female diagnosed with acute intermittent porphyria (AIP) at age 16 presented to our transplant center for consideration of orthotopic liver transplantation (OLT). She had the c517C > T exon T mutation in the porphobilinogen deaminase (*PBGD*) gene. During the first 2 years after her diagnosis she had several hospital admissions Due to her disease. She was subsequently treated with weekly hematin infusions and bimonthly phlebotomies to manage the resultant hyperferritinemia from her constant heme infusions. She reported a poor quality of life due to abdominal pain resulting in multiple hospital admissions. She also experienced significant fatigue and lethargy for several days following her hematin infusions. She described an average of 1–2 days of acceptable energy before experiencing symptoms again. Concern for her overall health and poor quality of life prompted her interest in liver transplantation as a cure for her disease. She felt that the risks of transplant and a lifetime of immunosuppression outweighed the risk of end organ damage and poor quality of life from her AIP.

The patient was evaluated for a liver transplant during a phase in her management marked by clinical stability. Prior to transplant, her urinary porphobilinogen (PBG) excretion was 269.7 mcmol/L, consistent with the diagnosis of AIP. She also had a serum ferritin level of 327 ng/mL, iron of 23 mcg/dL, and percent iron saturation of 4% (in the setting of regular phlebotomy). She underwent a partial liver transplant using the right lobe with middle hepatic vein and standard arterial anatomy. Her surgery was uncomplicated and she received empiric antibiotic coverage perioperatively (with micafungin and piperacillin-tazobactam). Transplant center protocol recommends the use of fluconazole, but micafungin was substituted given her history of AIP and uncertainty regarding kinetics of her PBG and aminolevulinic acid (ALA) clearance immediately post-op. Following transplantation, she had acute kidney injury and lactic acidosis, both of which resolved with hydration. Otherwise, she had an uncomplicated post-surgical experience.

The patient's immunosuppression regimen entailed our standard therapy triple protocol of tacrolimus, mycophenolate mofetil, and prednisone. Nebulized pentamidine, rather than sulfamethoxazole-trimethoprim, was used for pneumocystis pneumonia prophylaxis due to the potential risk for aggravation of neuropathy and abdomi-

Keywords: Acute intermittent porphyria**;** Liver transplant**;** Panhematin**;** Hemin.

Abbreviations: AIP, Acute intermittent porphyria; OLT, orthotopic liver transplantation; PBGD, porphobilinogen deaminase; PBG, porphobilinogen; ALA, aminolevulinic acid; ALAS, 5-aminolevulinic acid synthase; CEP, congenital erythropoietic porphyria; HCP, hereditary coproporphyria; HEP, hepatoerythropoietic porphyria; HMB, hydroxymethylbilane; PBG, porphobilinogen; PCT, porphyria cutanea tarda; VP, variegate porphyria; CYP 450, cytochrome P450; HAT, hepatic artery thrombosis.

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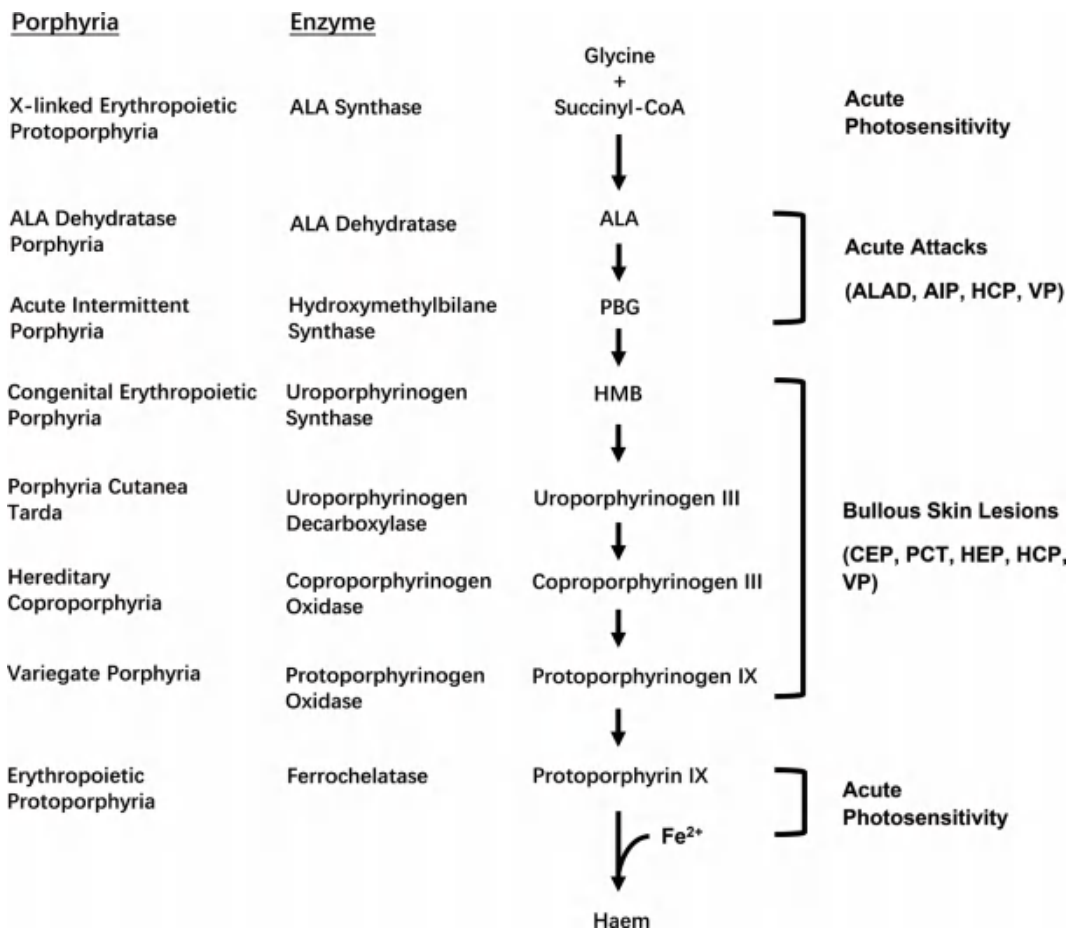

Fig. 1. Heme Biosynthesis Pathway. (*Adapted from Stein et al 2017***).** Abbreviations: AIP, acute intermittent porphyria; ALA, aminolevulinic acid; ALAD, ALA dehydratase; CEP, congenital erythropoietic porphyria; HCP, hereditary coproporphyria; HEP, hepatoerythropoietic porphyria; HMB, hydroxymethylbilane; PBG, porphobilinogen; PCT, porphyria cutanea tarda; VP, variegate porphyria.

nal pain resulting from the still present PBGD deficiency in nerve tissue peri-operatively. Since previous data notes a reported high rate of hepatic artery thrombosis in post-transplant AIP patients, our patient received therapeutic anticoagulation for a planned total course of nine months of treatment after transplant.

Nine months after transplant, the patient has shown no clinical or biochemical signs of porphyria and her liver allograft function is normal. She exhibits no clinical signs of neuropathy, neurologic impairment, or abdominal pain. She has been successfully weaned off previous pain medications, including gabapentin, and she now tolerates porphyria symptom-inducing medications. She reports a drastic improvement in her quality of life.

Given the success with OLT for the treatment of AIP, and resolution of biochemical markers and clinical symptoms, we present a summary of the pathophysiology, pharmacology and treatment options for AIP and a discussion of the current recommendations regarding liver transplantation as a cure for AIP.

Background

AIP is one in a group of inherited disorders that result from deficiencies in the heme biosynthesis pathway (Fig. 1). Mutations in the *PBGD*, which reduces the metabolic function of the enzyme, results in AIP.[1] The mutation presents as an autosomal disorder with low penetrance that depends on multiple factors, including environmental factors.[1,2] There are two broad categories of porphyria: the erythroid porphyrias and the hepatic porphyrias.[1] The hepatic porphyrias are further subdivided into four categories, including AIP, variegate porphyria, porphyria cutanea tarda and hereditary coproporphyria.[1,3] In this article, we provide a brief literature review of the epidemiology/pathophysiology of AIP, AIP diagnosis, precipitating factors, and treatment options, with an emphasis on liver transplantation as a curative treatment option.

Heme biosynthesis occurs in the erythroblastic system (80%) and liver (15%), as well as in other tissues (5%).[4] Regulatory mechanisms differ based on the site of production: erythroid heme synthesis depends mainly on the availability of iron, while hepatic heme synthesis is regulated by the free heme pool.[5] Heme synthesis begins with the formation of ALA within the mitochondria, catalyzed by 5-aminolevulinic acid synthase (ALAS). ALAS exists in two isoforms, namely the ubiquitously expressed ALAS1 and erythroid ALAS2.[5] The rate limiting step in hepatic haem synthesis is ALAS1 and this process is tightly regulated by intracellular haem which is the basis for the therapeutic effect of haemin in acute porphyria attacks.[5]

The first and final 3 steps of heme biosynthesis occur in the mi-

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tochondria, while the others are cytoplasmic. The first intermediate of the pathway is δ-ALA, which is formed by the condensation of glycine and succinyl CoA by δ-ALAS. Two molecules of ALA are then condensed by ALA dehydratase (ALAD) yielding monopyrrole—PBG.[4] Four molecules of PBG are combined by PBGD, also named hydroxymethylbilane synthase, yielding linear tetrapyrrole—hydroxymethylbilane, then converted to uroporphyrinogen III in the presence of uroporphyrinogen III synthase or spontaneously to uroporphyrinogen I. Decarboxylation of four groups of acetic acid to methyl groups of uroporphyrinogen III and I in the presence of uroporphyrinogen decarboxylase yields coproporphyrinogen III and I.[4] Coproporphyrinogen III oxidase then catalyzes oxidative decarboxylation of coproporphyrinogen III to protoporphyrinogen IX. In the presence of protoporphyrinogen oxidase, six atoms of hydrogen are removed from protoporphyrinogen IX to form protoporphyrin IX. The final stage of heme biosynthesis is the insertion of iron to the protoporphyrin IX ring and heme formation catalyzed by ferrochelatase.[4]

The *PBGD* gene is transcribed as two variants that are made by differential splicing of the primary gene transcript.[6] One splicing pattern, found specifically in erythrocytes, lacks exon 2 of the primary gene transcript, while the other is more ubiquitous and found in all cell types, especially in hepatocytes (hepatic form).[6] The liver is a major source of heme production, and decreased activity of the hepatic gene product form rather than the erythroid form causes buildup of toxic intermediates. These toxic intermediates are deposited in central, peripheral, autonomic, and enteric nervous systems. These toxic intermediates are thought to be responsible for many of the clinical symptoms observed in AIP.

Symptoms and clinical course

The majority of individuals with AIP present with nonspecific symptoms and many are asymptomatic. The clinical presentation is highly variable amongst those affected, often leading to a lack of clinical recognition and consequently, a delay in effective treatment. When symptoms present, they typically manifest as severe acute neuro-visceral attacks that can be life-threatening.[1,6] Attacks are the result of disease involvement in the central, peripheral, enteric and autonomic nervous systems.[1,7] When an episode develops, it occurs over 2 or more days; the severity and length of these attacks are related to precipitating factors and treatment. Patients are generally asymptomatic between attacks but may develop chronic symptoms over time.

The most common and earliest symptom of AIP is severe neuropathic abdominal pain that is diffuse in nature and often accompanied by nausea, vomiting, distention, constipation, and less often, diarrhea.[4,7] Symptoms may sometimes mimic those of acute appendicitis or gallbladder colic.[8] Long-term complications of the disease include chronic arterial hypertension, development of chronic renal disease, chronic liver disease, and hepatocellular carcinoma.[7,8]

Diagnosis

PBG is a precursor in the heme biosynthesis pathway and urinary levels of 20–200 mg/L during an attack of symptoms is diagnostic of AIP.[7] During remission, many AIP patients have PBG values 5–10 times the upper limit of normal (normal PBG reference range: <2 mg/L).[9] Hereditary coproporphyria and variegate porphyria differ from AIP in that PBG levels may not be as drastically increased with levels during an acute attack of at least 3- to 10-fold higher than normal and often normal at baseline.[7,8]. It is important to note that in patients already receiving treatment with hemin, PBG levels may be reduced, as hemin reduces the urinary levels of this precursor.[7] Other lab abnormalities may also be seen with AIP, such as increased serum levels of transaminases.[7] Diagnosis of AIP requires a high index of suspicion with uncommon and recurrent abdominal and neurological complaints. Biochemical testing is achieved through DNA testing or measurement of PBGD activity of erythrocytes; however, this should not delay initiation of treatment.[7]

Treatment

An important antecedent to initial therapy for AIP is the identification and withdrawal of any offending agents. Possible offending agents include certain medications, underlying disease, or changes in nutritional status. Once identified, correction may involve discontinuing the medication, substituting a therapeutic equivalent, treating the underlying disease, or correcting nutritional status with intravenous dextrose and maintenance fluids.

As stated previously, the inherent pathophysiology of porphyria is a result of increased porphyrin production from the result of overproducing ALA to achieve adequate levels of heme.[3,4,7,8] Several medications can contribute directly to the production of ALA and PBG, which leads to increased levels of porphyrin. Strong cytochrome P 450 (CYP 450) inducers and inhibitors can deplete the hepatic pool of heme, further driving feedback mechanisms to increase the heme supply by inducing the activity of ALAS, thereby directly increasing ALA and PBG, causing dysfunction of PBGD and increasing porphyrin levels.[4,10,11] It is difficult to assess if a medication will cause an acute porphyria attack, as many medications induce or inhibit CYP 450 isoenzymes to varying degrees. The American Porphyria Foundation and the European Porphyria Network have analyzed published case reports and added their expert opinions to this dilemma to create a list of medications and databases that can be used to determine if a medication can cause an acute porphyria attack.[4,7,12]

After identification and withdrawal of offending agents, the main goal of therapy for AIP is to extinguish an acute attack and to provide the patient with supportive care once an attack is abated. Currently, in addition to glucose, the mainstay of therapy is intravenous hemin.[13] This therapy is the only available treatment that is specifically labeled for acute porphyria exacerbations and takes anywhere from 2 to 5 days to resolve symptoms. It is thought that hemin represses the synthesis of and directly inhibits ALAS.[14–16] The hemin is prepared by crystallizing and recrystallizing hemin which is dissolved in 0.25% NazCO:1.[16] The dose varies and is initiated at a dosing range of 1 to 4 mg/kg with a maximum daily dose of 6 mg/kg and can be used for 3 to 14 days.[17,18] A rapid decline in porphyrin precursors in both blood and urine have been reported following hematin infusions, along with a complete remission of symptoms.[16]

Doses exceeding the maximum recommended can potentiate renal failure.[18] The main adverse reaction of hemin use is phlebitis. The chance of this occurring may be minimized through the administration of hemin via a slow-infusion central line. In addition, phlebitis may also be minimized by reconstitution of the hemin with albumin rather than sterile water. Another potential adverse event with hemin administration is the risk of iron accumulation with subsequent dosing for prophylaxis, as each 200 mg of hemin contains 17 mg of iron.[14–18]

Discussion

When other means of treatment are unsuccessful in reducing attacks and improving quality of life in AIP patients, liver transplantation can be considered, if the risks outweigh the benefits and the patient is deemed a suitable candidate for liver transplantation.[1,6] The type of porphyria must be considered, as documented benefit is only available for AIP, variegate porphyria, and erythropoietic protoporphyria. Of note, among the previously listed varieties of porphyria, patients with AIP are more likely to undergo liver transplantation. Historically, the first successful liver transplant for porphyria was performed in 2004 and several other successful cases have followed. Liver transplantation corrects the genetic deficiency of PBG deaminase, which leads to normalization of PBG and ALA levels. This results in resolution of symptoms for AIP and corrects the liver disease caused by variegate and erythropoietic porphyria.[6]

Dowman *et al*[19] reported a study in 2012 in which all liver transplants performed for AIP in the United Kingdom and Ireland between 2002 and 2010 were analyzed. In this analysis, liver transplantation was shown to be an effective treatment option. All patients who underwent liver transplant for AIP in this study were cured with resolution of both biochemical markers and symptoms. Transplanted patients demonstrated complete biochemical resolution with normalization of ALA and PBG levels within 24–72 hours of transplantation.[19] Improvement in quality of life was noted, including resolution of abdominal and neurovisceral attacks. Two deaths, both due to multi-organ failure post-transplant, were reported. Among these two deaths, one involved a patient who was ventilator-dependent for several months prior to the transplant.

The literature on AIP and liver transplantation notes that liver transplant does not result in improvement or cure of AIP among patients with longstanding neurological deficits and patients with severe neuromuscular or respiratory dysfunction. These types of patients have poorer outcomes, up to and including death.[19] While symptomatic benefit has been reported from AIP transplant, such complications as hepatic artery thrombosis (HAT), hemorrhage, renal dysfunction, bile leak and multi-organ failure may occur.[1,19] An analysis by Singal *et al*[1] reported that as of 2014, of the 14 known AIP liver transplant patients, bile leak was recorded for 1, renal dysfunction for 2, and hepatic artery thrombosis for 4. Yasuda *et al*[20] performed a review which showed that HAT was not seen at therapeutic levels of anticoagulation.

Patients with severe respiratory dysfunction, especially those requiring ventilation, may benefit from deferring transplant until they are successfully weaned from the ventilator.[21] While liver transplant cannot correct neurological impairment, performing liver transplantation prior to development of extensive neurological impairment may result in improved outcomes and quality of life post-transplant.

Conclusions

AIP is a rare and potentially life-threatening metabolic disorder that develops because of a deficiency in PBGD, a critical enzyme in the heme biosynthesis pathway. The disease typically manifests as painful acute neurovisceral attacks that can be triggered by numerous mechanisms, including certain medications. Treatment options include the use of IV hemin. However, symptoms do recur and ongoing disease and treatment can result in long-term damage to various organ systems, including the liver and kidneys. The goal of treatment is achievement of a definitive cure.

Here we describe the pharmacology of how medications contribute to attacks of AIP, the use of hemin to treat attacks, and the use of liver transplantation as a cure for AIP. While liver transplantation has been used to treat and cure AIP successfully, this option should be considered for cases of severe disease with recurrent bouts of attacks. Ideally, liver transplantation should be pursued prior to the patient developing significant end organ damage (such as to the kidney or lung) or significant neurological deficits. Issues to consider with liver transplantation include the risk of surgery, the risk of life-long immunosuppression, and the rate of HAT. The concern for HAT can be addressed with the use of anticoagulation for a period of time following transplantation. Therefore, liver transplantation should be considered as a viable option to cure patients with AIP.

Conflict of interest

The authors have no conflict of interests related to this publication.

Author contributions

Manuscript writing, critical revision, and technical or material support (TA, RW, BS, JB, MK, SC, JW).

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Review Article

Phytochemical Composition and Pleotropic Pharmacological Properties of Jamun, *Syzygium Cumini* Skeels

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Abstract

Plants have been employed as medicine since time immemorial, and there has been a recent resurgence in the use of plants as medicines due to their little or no toxicity at the doses used for treatment of different ailments. This review discusses in detail the phytochemical and pharmacological activities of Jamun (*Syzygium cumini*), a tree belonging to family Myrtaceae, which has been credited with several medicinal properties in the traditional system of medicine, the Ayurveda. The different properties attributed to Jamun are sweet, sour, astringent, acrid, refrigerant, carminative, diuretic, and digestive. Research and practical use in traditional medicinal systems have found Jamun to be effective in treating leucorrhoea, gastric disorders, fever, diabetes, piles, stomachache, wounds, and dental, digestive and skin disorders. Some compounds in Jamun have antioxidant, antimicrobial, antiallergic, antidiabetic, antihyperlipidemic, anticancer, gastroprotective, hepatoprotective, cardioprotective and radioprotective activity. Finally, Jamun has been found to contain phytochemicals including anthroquinones, alkaloids, catechins, flavonoids, glycosides, steroids, phenols, tannins, saponins and cardiac glycosides. The diverse activities of Jamun may be due to its abilities to scavenge free radicals, increase antioxidant status of cells by increasing glutathione, glutathione peroxidase, catalase and/or superoxide dismutase, and to attenuate lipid peroxidation. In addition, it also suppresses the transcription of peroxisome proliferator-activated receptor, Nuclear factor kappa B, cyclooxygenase, inducible nitric oxide synthase, tumor necrosis factor alpha and other proinflammatory cytokines, accompanied by the up-regulation of nuclear factor erythroid 2-related factor 2 transcription, which is involved in regulating the antioxidant status of the cells.

Introduction

Taxonomically, Jamun belongs to Kingdom: Plantae; Division: Magnoliophyta; Class: Magnoliopsida; Order: Myrtales; Family: Myrtaceae; Genus: *Syzygium* and Species: *cumini*. It is also known by the other names of *Syzygium jambolana* (Lam.) DC, *Syzygium jambolanum* DC, *Syzygium caryophyllifolium* (Lam.) DC, *Calyptranthes oneillii* Lundell, *Calyptranthes jambolana* Willd., *Eugenia cumini* Druce, *Eugenia caryophyllifolia* Lam., *Eugenia jambolana* Lam., *Eugenia djouat* Perr., and *Myrtus cumini* L (Fig. 1). It is native to the Indian subcontinent, and is widely distributed throughout India, Pakistan, Bangladesh, Myanmar and Ceylon. However, Jamun has been introduced into different parts of the world, including the United States of America, for its economic importance as a producer of fruits and timber.

Jamun is a fast growing tree that reaches heights up to 100 feet, and it bears fruits in clusters during the summer. Each fruit cluster may contain fruits numbering only a few to as many as 10 or even 40. The Jamun fruits are round to oblong in shape, with the size of each varying between 1/2 to 2 inches (Fig. 1). They are green in color and turn from light to dark purple, or even black coloration, once they are fully ripe.[1] The taste of the Jamun fruits is sweetish sour, and eating of the fruits tends to color the tongue purple.

The Jamun tree is considered sacred by Hindus and Buddhists, alike, and is commonly found in the compounds of Hindu temples. It is considered dear to Lord Krishna and its leaves and fruits are offered to Lord Ganesha (Elephant God) during his worship.[2] The Jamun tree is also known by other names across the globe, such as black plum, Indian blackberry, Jaman, Jambu, Jambul, Jambool,

Keywords: Jamun; Medicine; Antioxidant; Antidiuretic; Radioprotective; Antihyperlipidemic.

Abbreviations: PPAR, peroxisome proliferator-activated receptor; NF-κB, Nuclear factor kappa B; COX, cyclooxygenase; iNOS, inducible nitric oxide synthase; TNFα, tumor necrosis factor alpha; Nrf2, nuclear factor erythroid 2-related factor 2; NO, Nitric Oxide; OH, Hydroxyl; DPPH, 2,2-diphenyl-1-picrylhydrazyl; O_2^{-}•, superoxide; FRAP, ferric reducing power; ABTS, 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid; DCM-MET, dichloromethane and methanol; HDL-c, high density lipoprotein cholesterol; LDL-c, low density lipoprotein cholesterol; GSH, glutathione; SOD, superoxide dismutase.

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Fig. 1. Different parts of Jamun, *Syzygium cumini.* a: leaves; b: stem; c: fruit; d: seed.

Java plum, Malabar plum, Duhat and Portuguese plum.[3,4]

Traditional medicinal uses

Plants have been used by humans for healthcare since the advent of human history, and they are still used by the majority of the population due to their non-toxic nature and economic affordability. However, the scientific basis of use of these medicinal plants still needs to be validated. Jamun is one of the medicinal plants that have been used in diverse traditional ethnomedicinal practices to treat various disorders in humans.[5] Jamun's most common uses have included diabetes and in dental, digestive, liver and skin disorders.

In Ayurvedic medicine, 1–3 g of dried seed powder is usually given orally to humans to treat diabetic conditions. Different parts of Jamun have been used to cure blisters in the mouth, colic, digestive complaints, diarrhea, dysentery, diabetes, pimples, piles, stomachache, and cancer.[6] Juice of the ripened fruit is administered in the dose range of 0.5–2 teaspoons (2.5–10 mL) thrice a day to treat diabetes in humans.[7,8] Jamun is also considered a good general health tonic for humans, acting as a blood purifier. The Jamun stem bark is used as an astringent, anthelmintic, antibacterial, carminative, constipating, diuretic, digestive, febrifuge, refrigerant, stomachic and sweet. The fruits and seeds are used to treat asthma, diabetes, bronchitis and splenopathy.[9,10] Fresh Jamun fruit pulp with honey is administered to keep the body healthy, while its seed powder is given to help in clearing the skin blemishes left by blackheads and acne. The fruit juice is also useful for treating enlarged spleen and resolving urinary problems.[11] Jamun seed powder mixed with jaggery provides relief in diarrhea and dysentery. The leaf juice and poultice of the leaves are effective in the treatment of dysentery and skin disorders.[12,13] Moreover, the application of Jamun leaf ash cures bleeding gums and keeps teeth healthy,[8] while the leaf paste is a good general wound healing agent.

In Unani medicine, Jamun is used as a liver tonic. And, similar to its application in Ayurvedic medicine, Jamun is known in Unani medicine to strengthen teeth and gums, enrich blood, and deworm against ringworm infection of the head.[14] The Jamun fruit pulp is also used to treat gingivitis, and application of Jamun for 2–4 months is helpful in treating hemorrhoids.

The Jamun stem bark, dried seeds and root bark decoction is used to treat diarrhea, dysentery and dyspepsia, and it can also act as an enema.[13] Stem bark powder mixed with yoghurt is given to treat menorrhagia,[7] and when mixed with Jamun fruit juice cures cough and cold. In addition, one glass of Jamun fruit juice with half teaspoon of stem bark powder given daily relives the problems of urinary tract disorders and urinary infections. In India, the Jamun seed powder is used as an antidote against strychnine poisoning.[12] The decoction of the Jamun stem bark is used to cure asthma and bronchitis.[15] When the stem bark decoction is gargled or used as a mouthwash it can cure mouth ulcerations, spongy gums and stomatitis.[11,13] The stem bark ashes of Jamun can either be mixed with water and used as a general anti-inflammatory agent, or mixed with oil and used to treat burns.[11] Seed decoction of Jamun relieves fatigue and strain.

Research into the traditional uses of Jamun has encompassed investigations into the application of all different parts of the Jamun plant by means of various study systems, with the aim of substantiating the claims of traditional healers and harnessing its diverse medicinal properties for evidence-based modern clinical practice.

Phytochemical analysis

The medicinal properties of Jamun may be due to its ability to synthesize various phytochemicals. Indeed, many investigators have studied the phytochemical profiles of Jamun roots, stem, leaves and fruits, and their findings are detailed in this section.

The leaves of Jamun have been extracted in methanol and water, and analyzed for the presence of different phytochemicals. Both aqueous and methanol extracts have been found to possess a range of alkaloids, flavonoids, glycosides, steroids, phenols, tannins, saponins and cardiac glycosides.[16,17] Meanwhile, the ethanol stem bark extracts of Jamun have shown the presence of terpenoids, alkaloids, catechins, phenols, quinones, saponins and tannins, whereas, the methanol extract of such was found to additionally contain flavonoids. On the other hand, the aqueous stem bark extract only contained catechins, phenols, quinones and flavonoids.[18] Likewise, the ethyl acetate and methanol extracts of Jamun seeds were found to contain flavonoids, alkaloids, glycosides, triterpenoids, steroids, saponins, and tannins.[19]

Different leaf extracts of Jamun have been tested for the presence of various phytochemicals, and the methanol, ethanol and aqueous extracts of leaves were found to contain flavonoids, anthroquinones, tannins, phenols, steroids. All except the aqueous extract contained alkaloids. The analysis of chloroform and petroleum ether extracts showed the presence of flavonoids anthroquinones and steroids, but a striking absence of tannins, terpenoids, saponins, phenols and alkaloids. The cardiac glycosides were absent in all the extracts of Jamun leaf reported.[20]

The phytochemical analysis of ethanol extract of Jamun stem bark, leaf, seed and fruit pulp showed the presence of alkaloids, anthroquinone glycosides, flavonoids, tannins, saponins, phenols, cardiac glycosides, terpenoids, phytosterols, steroids and amino

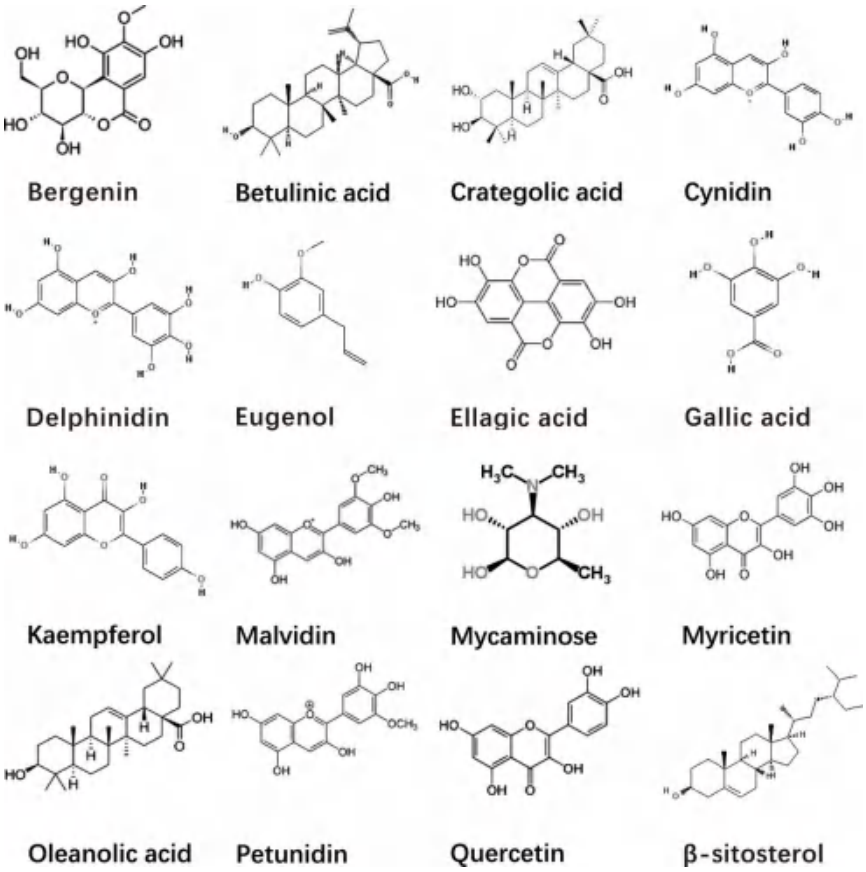

Fig. 2. Chemical structures of some important phytochemicals present in different parts of Jamun, *Syzygium cumini.*

acids in all extracts, with the exception that anthroquinones were absent in the seed and pulp extracts, whereas terpenoids and phytosterols were absent in the leaf extract.[21]

Active components

The different parts of Jamun have been subjected to isolation and characterization of various types of phytochemicals. including the flavonoids, phenolic acids, terpenes, tannins and anthocyanins (Fig. 2). The leaves of Jamun were found to contain betulinic acid, crategolic acid, n-dotricontanol, n-hentriacontane, n-hepatcosane, mycaminose, myricetin, myricitrin, myricetin 3-O-(4″-acetyl)-α-L-rhamnopyranosides, n-nonacosane, quercetin, β-sitosterol, noctacosanol, n-triacontanol, triterpenoids, tannins, eicosane, octacosane and octadecane.[1, 22–25] The essential oils from Jamun leaves showed the presence of α-cadinol, geranyl acetone, muurolol, α-myrtenal, pinocarvone, pinocarveol, a-terpeneol, myrtenol, eucarvone, cineole, alloocimene, α-bornyl acetate, α-pinene, 2-β-pinene, caryophyllene, caryophyllene oxide, L-limonene, α-humulene, α-terpineol and α-terpineolene.[26–28]

The flowers of Jamun have shown the presence of various phytochemicals, including kaempferol, quercetin, myricetin, isoquercetin (quercetin-3-glucoside), myricetin-3-L-arabinoside, quercetin-3-D-galactoside, dihydromyricetin, oleanolic acid, acetyl oleanolic acid, eugenol-triterpenoid A and eugenol-triterpenoid B.[29]

The fruit of Jamun consists of different anthocyanins (including delphinidin 3,5-diglucoside, cyanidin 3,5-diglucoside, petunidin 3,5-diglucoside, peonidin 3,5-diglucoside, delphinidin 3-glucoside, malvidin 3,5-diglucoside, delphinidin acetyl-diglucoside, peonidin-3,5-diglycoside, petunidin 3-glucoside, malvidin 3-glucoside), non-anthocyanic phenolic compounds [such as galloyl-glucose ester, ellagic acid and gallic acid (phenolic acid)], and flavanols (such as dihydroquercetin diglucoside, dihydromyricetin diglucoside-methyl-dihydromyricetin, diglucoside, dimethyl-dihydromyricetin diglucoside, myricetin glucoside, myricetin pentoside, myricetin rhamnoside , myricetin acetyl-rhamnoside and myricetin).[30–32] The fruit pulp also includes cyanidin 3-glucoside, cyanidin 3-rutinoside, cyanidin 3-xyloside, cyanidin 3-malonylglucoside, cyanidin 3-dioxalylglucoside, quercetin 3-rutinoside, quercetin 3-galactoside, quercetin 3-glucoside, quercetin 3-glucuronide, quercetin 3-O-[600-(3-hydroxy-3-methylglutaroyl)]-β-galactoside, quercetin 3-glucosylpentoside, quercetin 3-oxalylpentoside, quercetin 3-rhamnoside, quercetin, lambertianin C isomer, sanguiin H-6 lambertianin A and galloyl-bis-HHDP glucose isomer.[28,33]

The seeds of Jamun have been reported to include 7-hydroxy-calamenene, methyl-β-orsellinate, β-sitosterol, and oleanolic acid, and 3-hydroxy androstane [16,17-C](6′methyl, 2′-1-hydroxy – isopropene-1-yl) 4,5,6 H pyran.[34] The methanol extract of Jamun seeds showed the presence of 34 chemicals, principle among them being 5,10-dichloro-5,10-dimethyltricyclo[7.1.0.0(4,6)]decane, tetradecanoic acid, α-caryophyllene, 1,10-decandiol, β caryophyllene, bicyclo(4.4.O)decane, octadienol, cadinene, 4-dodecen-1-ol acetate, 2-furancarboxaldehyde 5-(hydroxymethyl) and oxirane 2,3-dimethyl. The ethanol extract showed presence of caryophyllene oxide, bicyclo(7.2.0)undec-4-ene, 4,11,11-trimethyl-8-methylene,

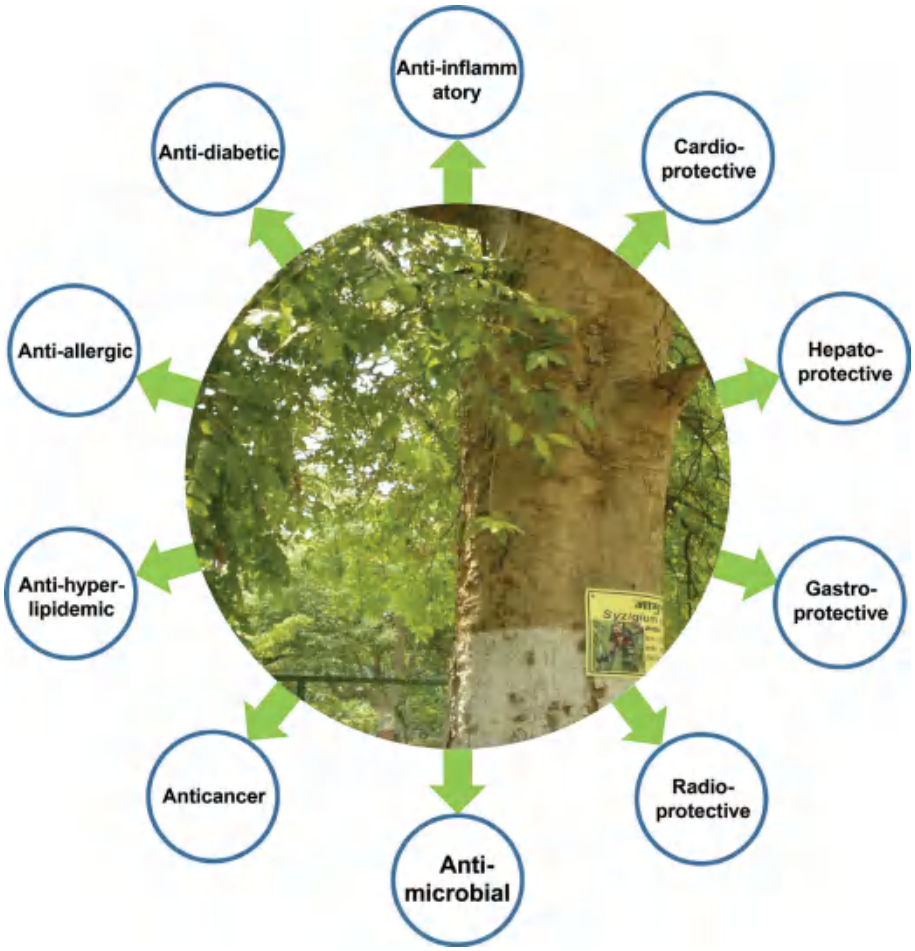

Fig. 3. Different activities of various parts of Jamun, *Syzygium cumini.*

5(hydroymethyl)-2-furaldehyde, isogeraniol, 3(2H)-furanone dihydroxy-2-methyl, decahydro-4A-methyl-1-methylene-7-(1-methylethenyl), 12-methyl-E,E-2,13-octadecadien-1-ol, nondecanoic acid, guaiol, limonene oxide, 2-methyl-3-isobutenyl-4-penten-2-ol, 3-methyl-4-hexyn-3-ol,thujanol and 10-undecyn-1-ol.[35]

The stem bark has been found to contain betulinic acid, friedelin, epi-friedelanol, β-sitosterol, eugenin and fatty acid ester of epi-friedelanol.[36] β-sitosterol, quercetin kaempferol, myricetin, gallic acid and ellagic acid, bergenins, flavonoids and tannins have also been reported from the stem of Jamun.[37–39]

Antioxidant activity

Various parts of Jamun have been shown to exert antioxidant activity, as indicated by free radical scavenging assays. Moreover, the Jamun leaf and seed extracts have shown a concentration-dependent increase in the scavenging activity of nitric oxide (NO) free radicals.[40] The aqueous extract of Jamun fruit skin has been found to scavenge hydroxyl (OH), superoxide (O_2^{-}•) and DPPH free radicals.[41]

The DPPH radical scavenging and ferric-reducing power (FRAP) of leaf extract were evaluated in the methanol extract and its fraction of ethyl acetate, chloroform, n-hexane and water. The ethyl acetate fraction was found to be most potent in scavenging of DPPH radicals and FRAP.[42] The anthocyanin-rich extract prepared in acidified (5% H_3PO_4) ethanol from Jamun fruits was shown to scavenge 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) anion and peroxy radicals efficiently.[31]

The 1:1 dichloromethane and methanol (DCM-MET) extract of Jamun leaves was evaluated for its ability to scavenge different free radicals *in vitro*. The Jamun extract was found to scavenge OH free radicals in a dose-dependent manner and maximum effect was achieved with 350 µg/mL. This extract also inhibited the generation of O_2^{-}• radicals, and the greatest effect was achieved with 250 µg/mL. Similarly, it equally inhibited DPPH and $ABTS^{+}$ free radicals, and the highest effect was achieved with 80 µg/mL for both radicals.[43]

The acid ethanol extracts of seed and fruit pulp of Jamun have been reported to inhibit generation of DPPH and $ABTS^{+}$ free radicals, and to exhibit iron chelating activity.[44] The aqueous leaf extract of Jamun was shown to scavenge OH, O_2^{-}•, NO and $ABTS^{+}$• free radicals in a concentration-dependent manner.[45] The methanol, and methylene chloride extracts of Jamun leaves and its oils have been reported to scavenge DPPH radicals and increase the FRAP.[46] The studies on methanol and aqueous leaf extracts of Jamun have also revealed an ability to inhibit the generation of OH, NO, and DPPH free radicals in a concentration-dependent manner and to cause an increase in the FRAP with increasing concentrations.[47]

The above-mentioned DPPH radical scavenging and FRAP

Table 1. Antiinflammatory and antiallergic activities of Jamun, *Syzygium cumini*

S. No.	Parts used	Extract type	Activity	Species	References
1.	Seed	Chloroform	Anti-inflammatory	Rat	[55]
		Methanol &ethyl acetate		Rat	[58]
		Aqueous		Human	[56]
2.	Stem bark	Ethanol		Rat	[57]
3.	Leaf	Methanol		Rat	[59]
		Essential oil		Rat Mice	[60] [61]
		Aqueous		Mice	[45]
4.	Flavonoid fraction	—		Human	[62]
5	Root	Aqueous		RAW 264.7 macrophages	[54]
6	Leaf	Aqueous	Antiallergic	Mice	[63]
	Root	Aqueous Methanol		Mice	[64]

study revealed that the ethanol seed extract was the most potent, followed by stem bark and leaf extracts of Jamun.[21] In another study, the methanol leaf extract was shown to increase the FRAP with increasing concentration and demonstrated its superiority to fruit pulp and seed extracts.[48]

Antibacterial and antifungal activity

Essential oils extracted from the Jamun leaves have been reported to exert antibacterial properties against *Basillus sphaericus, Basillus sphaericus, Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa* and *Samonella typhimurium*.[26] The hydroalcoholic extract of Jamun leaves was found to be active against *Candida krusei* and antibiotic-resistant bacterial species of *P. aeruginosa, Klebsiella pneumoniae* and *S. aureus* in addition to *Enterococcus faecalis, E. coli, Kocuria rhizophila, Neisseria gonorrhoeae, P. aeruginosa*, and *Shigella flexneri*.[49] The diethyl ether, methanol and aqueous extracts of Jamun fruit inhibited the growth of *Bacillus cereus, Staphylococcus epidermidis, Micrococcus luteus* and *Salmonella typhi*, respectively.[50] The ethanol extract of Jamun leaf has been reported to be active against the *Vibrio cholerae* serogroups Ogawa and Inaba.[51]

The 70% ethanol extract of Jamun leaf, bark, pulp and seed showed a potent antimicrobial activity against various Gram-positive bacteria (including *B. subtilis, B. cereus* and *S. aureus*) and Gram-negative bacteria (including *S. flexneri, P. aeruginosa* and *V. cholera*). Comparison analysis showed that the leaf and bark extracts were more potent than the pulp and seed extracts.[21] The ethanol extract of Jamun seeds was found to inhibit the growth of *E. coli, B. subtilis, P. aeruginosa* and *S. aureus*.[52] The aqueous extract of Janum stem and leaf showed antibacterial activity against all strains of bacteria, including *S. aureus, Staphylococcus saprophyticus, E. coli, P. aeruginosa* and *Proteus vulgaris*, whereas the fruit extract was active against *P. aeruginosa* only and the maximum antifungal activity was recorded for *Penicillium chrysogenum* and *Candida albicans*.[53]

The methanol and methylene chloride leaf extracts and the essential oils from Jamun leaves exhibited antibacterial activity against both Gram-positive and Gram-negative bacteria, and the methanol extract was superior to the methylene chloride extract and leaf oil.[46] The ethanol extract of Jamun roots was found to be active against *S. aureus, S. epidermidis, E. coli, Streptococcus suis, Salmonella spp*. and *Corynebacterium diphtheriae*. The root extract of Jamun was found to be more effective against Gram-positive bacteria than Gram-negative bacteria.[54]

Anti-inflammatory activity

Jamun has been reported to act as an anti-inflammatory agent, reducing both acute and chronic inflammation (Fig. 3 and Table 1[45, 54–64]). The chloroform seed extract has been reported to inhibit carrageenan (acute), kaolin-carrageenan-induced paw edema in the rats and to also suppress protein exudates, leakage of dye in peritoneal inflammation, and leukocyte migration.[55] Similarly, the aqueous seed extract was found to exert an anti-inflammatory effect against human neutrophils.[56]

Preclinical studies using animal models have shown that the ethanol extract of Jamun stem bark exhibits anti-inflammatory activity, as demonstrated in carrageenan (acute), kaolin-carrageenan (sub-acute) and formaldehyde (sub-acute)-induced paw edema, as well as cotton pellet granuloma (chronic) rat models.[57] The methanol and ethyl acetate seed extracts have shown an anti-inflammatory response in carrageenan-induced rat paw edema.[58] The methanol extracts of Jamun leaves have also been shown to reduce acute and chronic inflammation in carrageenan, histamine and serotonin-induced rat paw edema and cotton pellet-induced rat granuloma studies.[59] In another study, essential oils from the Jamun leaf inhibited the migration of rat eosinophils, indicating that Jamun leaf possesses anti-inflammatory activity.[60]

The aqueous leaf extract has also been reported to reduce indomethacin-induced inflammatory changes in the mice by reducing nitric oxide synthase (iNOS), tumor necrosis factor-alpha (TNF-α) and cyclooxygenase (COX) enzymes.[45] The essential oils from Jamun leaf have been reported to alleviate chronic granulomatous inflammation in BALB/c mice that had been intravenously infected with *Mycobacterium bovis* Bacillus Calmettte-Guerin.[61] The fla-

Table 2. Protective effect of Jamun, *Syzygium cumini* on different disorders

S. No.	Parts used	Extract type	Activity	Species	References
1.	Leaf	Aqueous	Hepatoprotective	Rat	[66]
2.	Fruit	Ethanol Methanol		Rat Rat	[30] [67,68]
3.	Seed	Ethanol		Rat	[69]
4.	Stem		Gastroprotective	Rat	[70]
5.	Seed	Ethanol		Rat	[71,72]
6.	Seed	Methanol	Cardioprotective	Rat cardiomyocytes	[35,73,76]
		Ethanol		Rat	[74]
		Powder		Human	[75]
	Leaf	Hydroalcoholic		Rat	[77]

vonoid fraction of Jamun has been reported to alleviate inflammatory response in human lymphocytes, monocytes and neutrophils against the hepatitis B vaccine.[62] The aqueous and ethanol extracts of Jamun root were shown to reduce IL-6 production in RAW 264.7 macrophages, indicating slight anti-inflammatory activity.[54] The aqueous seed extract was found to exert anti-inflammatory activity in diabetic rats, as indicated by the suppression of ectonucleotidase, adenosine deaminase, acetyl cholinesterase, dipeptidyl peptidase IV and NO activities.[65]

Antiallergic activity

The antiallergic effect of aqueous leaf extract of Jamun has been investigated in mice that had been injected with the mast-cell degranulator C48/80 or with ovalbumin (OVA) to induce anaphylaxis edema (Fig. 3). Treatments at various doses of Jamun extract was found to reduce the edema; no significant difference was found between the different doses used (Table 1). The treatment of rats with C48/80 released histamine in peritoneal mast cells, whereas pretreatment with Jamun leaf extract (1 µg/mL) inhibited this allergic reaction in the mast cells. Administration of OVA to BALBc mice induced substantial accumulation of leukocytes, mononuclear cells and eosinophils in the pleural cavity, whereas pretreatment of these mice with Jamun leaf extract at 1 h before the OVA administration significantly suppressed the accumulation of eosinophils in the pleural cavity, indicating its anti-inflammatory action.[63] Moreover, study of the antiallergic effects of aqueous, methanol and methanol fraction of the aqueous extract of Jamun roots revealed that these extracts suppressed clonidine-induced catalepsy in mice by inhibiting the release of histamine triggered by mast cell degranulation. Finally, the administration of different root extracts of Jamun was shown to suppress milk-induced eosinophilia in mice.[64]

Hepatoprotective activity

Administration of aqueous leaf extract of Jamun to albino rats for 7 days prior to carbon tetrachloride treatment has been found to be hepatoprotective (Table 2[30, 35, 66–77]), as indicated by the alleviation of enhanced levels of aspartate aminotransferase and alanine aminotransferase compared to control rats treated with carbon tetrachloride alone.[66] The fruit extract of Jamun has also been reported to protect rat hepatocytes against carbon tetrachloride-induced toxicity *in vitro*.[30] Similarly, the administration of ethanol extract of Jamun fruit pulp for 8 consecutive days prior to paracetamol treatment has been shown to protect rats against paracetamol-induced hepatotoxicity.[67] The Jamun fruit extract has also been found to reduce bile duct ligation-induced damage to hepatocytes, hepatic fibrosis and macrophage infiltration by reducing lipid peroxidation and mRNA expression of intracellular adhesion molecule (Icam-1) and Chemokine (C-X-C motif) ligand 2 (Cxcl2 genes). The fruit pulp extract of Jamun was also shown to reduce NO production by suppressing iNOS transcription, as well as transcriptional activation of NF-κB.[68] The aqueous seed extract has been found to protect against liver damage in streptozotocin-induced diabetic rats.[78] In addition, rats administered with seed extract for 14 days prior to carbon tetrachloride administration were protected against the carbon tetrachloride-induced hepatotoxicity.[69] The findings from all of the above studies indicate the hepatoprotective potential of Jamun (Fig. 3).

Gastroprotective

Tannins extracted from the stem bark of Jamun protect against gastric ulcers (Table 2) in Sprague-Dawley rats induced by oral administration of HCl/ethanol, as indicated by alleviated gastric mucosal damage, reduced free radicals and reduced ulceration of the gastric mucosa.[70] In addition, the ethanol extract of Jamun seeds has been reported to reduce indomethacin- and ethanol–induced peptic ulcers and acid-pepsin output in the streptozotocin–induced diabetic rats.[71,72]

Cardioprotective activity

Cardiovascular disorders represent the number one killer disease in the world, and the different extracts of Jamun have been investigated in diverse preclinical models for their cardioprotective activity (Table 2). Methanol extract of Jamun seeds administered orally at 500 mg/kg daily for 30 days to isoproterenol-treated rats was able to protect against myocardial damage.[73] The ethanol extract of Jamun seed powder administered to Wistar rats for subsequent 15 days at 1 hour before doxorubicin treatment, in a similar fashion, protected cardiac tissues against the doxorubicin–induced cardiotoxicity.[74]

A randomized, double-blind, placebo controlled clinical trial of

Table 3. Antidaibetic and antihyperlipidemic effects of different extracts of Jamun, *Syzygium cumini*

S. No.	Parts used	Extract type	Activity	Species	References
1.	Seed	Aqueous	Antidiabetic	Rabbits Rat	[80] [81–87]
		Powder		Rat Human Mice	[92,98,99] [75,101] [88,100]
		Ethanol		Rat and Rabbit Rat	[89,90,93] [71,72]
		Ethyl acetate Methanol		Rat	[58] [58,73,99]
2.	Stem	Ethanol		Rats	[85,86,99]
3.	Fruit pulp	Lyophilized		Rat	[93]
		Aqueous Ethanol		Rat	[94,96]
4.	Leaf	Aqueous		Humans Rat	[95] [99]
5.	Seed	Aqueous	Hyperlipidemia	Rabbit	[89]
		Ethanol		Rat Mice	[91,96–98,100,102] [100]
6.	Fruit	Aquoeus		Rat	[96]
		Ethanol		Rat	[102]

99 diabetic patients given 5 g of seed powder before meals twice daily for 90 days has been reported, and the results indicate the powder lowered blood pressure and exerted a hypoglycemic action.[75] The methanol seed extract of Jamun has also been found to protect H9C2 cardiomyoblasts against glucose-induced stress.[35,76] The hydroalcoholic extract of Jamun leaves, orally administered at a dose of 0.5 g daily for 8 weeks to hypertensive rats, has been reported to reduce hypertension.[77] These collective preclinical and clinical model studies indicate that Jamun also acts as a cardioprotective agent (Fig. 3).

Antidiabetic activity

Diabetes afflicts a large number of the world's population, and Indians are especially prone to it. Despite the fact that this ailment was uncommon in ancient times, Ayurveda pharmacopeia mentions the antidiabetic effect of Jamun, whereby its seed powder is reported to control high blood sugar levels (Fig. 3). In the Western world, Jamun has been applied as a treatment to control blood sugar levels for more than 130 years now; however, clinical studies have shown mixed results. While some patients responded well, others did not respond to the treatment at all.[79] The antidiabetic activity of Jamun has also been investigated in several preclinical animal models, which have demonstrated hypoglycemic effects for the different parts of Jamun (Table 3[58, 71–73, 75, 80–102]).

In the twentieth century, a report was published citing no evidence of any reduction in blood sugar levels in alloxan-induced diabetic rats fed with Jamun seed extract.[103] Thereafter, numerous reports were published in which various animal models showed evidence of Jamun reducing blood sugar levels. The ethanol extract of Jamun seed was reported to reduce blood glucose levels in streptozotocin-induced diabetes in rats[71,72]; and, the methanol extract of Jamun seeds was reported to reduce serum glucose level in the alloxan-induced diabetes in rats.[75] The aqueous seed extract of Jamun has been reported to reduce blood glucose level in both diabetic rabbits and rats.[80–84] The isolated compound mycaminose and ethyl acetate and methanol extracts of Jamun seeds also reduced blood glucose level in the streptozotocin-induced diabetic rats.[58]

The stem bark extract of Jamun was also found to decrease blood glucose levels in spontaneous diabetic rats.[85,86] The aqueous lyophilized seed powder was effective in lowering the blood glucose level in both diabetic mice and rats.[87,88] Interestingly, the water soluble seed extract containing gummy fibers was effective in controlling diabetes in alloxan-induced diabetes in rats, whereas the aqueous extract without gummy fiber was completely ineffective.[104] The ethanol extract of Jamun seeds has been shown to reduce fasting blood glucose levels in the alloxan or streptozotocin-induced diabetes in rats and rabbits.[89]

Similarly, the ethanol extract of seed kernel depleted levels of blood glucose, urea and cholesterol, increased glucose tolerance and reduced the glutamate oxaloacetate transaminase and glutamate pyruvate transaminase, and it also restored the activities of superoxide dismutase (SOD), catalase and glutathione peroxidase enzymes, and reduced glutathione (GSH) contents in liver and kidney of streptozotocin-induced diabetic rats.[90,91] Streptozotocin-induced rats fed with different doses of Jamun seed powder have shown a reduced fasting glucose level, indicating that the administration of Jamun seed powder was highly effective in controlling diabetes.[92] However, in a Brazilian study, treatment of streptozotocin-induced diabetic rats with the lyophilized fruit pulp extract of Jamun was unable to reduce the raised blood sugar levels.[93]

The effect of aqueous and ethanol extracts of Jamun fruit pulp was studied in the alloxan-induced diabetic rats and both extracts

were found to alleviate the blood glucose level, but the aqueous extract was more potent than the ethanol extract.[94] Aqueous leaf extract of Jamun has also been reported to reduce the glucose levels, as well as the adenosine deaminase levels, in the serum of diabetic patients.[95] The aqueous extract of the Jamun fruit pulp has been reported to reduce serum glucose level in streptozotocin-induced diabetes in female Wistar rats; however, the combination of Jamun fruit extract with the stem bark extract of *Cinnamon zeylanicum* was more effective than the either treatment alone.[97] The active components isolated by sephadex gel chromatography from the ethanol fraction of Jamun seed extract have been shown to reduce glucose level in the serum of alloxan-induced mild and severe diabetes in rats.[97] Moreover, treatment of streptozotocin-induced type–II diabetic rats with 400 mg/kg aqueous seed extract of Jamun brought the glucose level to near normal and also increased the expression of PPARγ and PPARα proteins in the rat liver.[98]

The aqueous and methanol extracts of root, stem bark, leaf and seed of Jamun have been reported to lower serum glucose levels in alloxan-induced diabetes in male Sprague-Dawley rats.[99] The aqueous extract of Jamun seeds has also been reported to reduce serum glucose levels in alloxan-induced diabetes in mice.[100] In human diabetic subjects, supplementation of Jamun seed powder for 30 days reduced the fasting and post-prandial blood glucose levels.[101] The results from these collective studies indicate that Jamun seeds possess the ability to control blood sugar in the diabetic condition (Table 3).

Antihyperlipidemic activity

Diabetes is often accompanied by hyperlipidemia, which results in cardiovascular morbidity, and different parts of Jamun have been investigated for cholesterol lowering activity (Fig. 3 and Table 3). The seed extract of Jamun has been reported to decrease total serum cholesterol:high-density lipoprotein (HDL) cholesterol ratio, serum low-density lipoprotein (LDL) cholesterol levels and to reduce the activity of HMG-CoA reductase in alloxan-induced diabetic rabbits.[89] Jamun has both the ability to lower hyperglycemia as well as the enhanced lipid contents. The seed kernel ethanol extract of Jamun has been reported to reduce the LDL and very low-density lipoprotein cholesterol levels in streptozotocin-induced diabetic rats; whereas, HDL cholesterol was increased, indicating that Jamun seed extract possesses antihyperlipidemic activity.[92]

The aqueous fruit pulp extract of Jamun has been found to alleviate enhanced triglycerides and total cholesterol, and to increase HDL cholesterol in the streptozotocin-induced diabetic rats.[96] Likewise, the active components separated by sephadex gel chromatography from the ethanol seed extract fraction of Jamun have been reported to alleviate enhanced triglycerides and total cholesterol, and to increase HDL cholesterol in alloxan-induced diabetic rats.[97] Aqueous seed extract of Jamun reduced the hyperlipidemic effect in alloxan-treated mice, by alleviating the enhanced levels of triglycerides, increasing the HDL cholesterol level, and returning the total cholesterol to normal.[100] Finally, the ethanol extracts of seeds and fruits of Jamun have been reported to decrease the enhanced levels of triglycerides and LDL and to increase the HDL cholesterol level in rats fed with a high cholesterol diet.[102]

Radioprotective Activity

Every human and animal is exposed to ionizing radiation in daily life, from cosmic sources, air and space travel and diagnostic or medical treatment. Ionizing radiation is harmful and poses various threats to health, such as induction of cardiovascular, pulmonary, liver, kidney and reproductive disorders and cancer. This necessitates the search for pharmacological agents that can protect against the deleterious effects of ionizing radiation.

The radioprotective activity of Jamun leaf and seed extracts (Fig. 3) was evaluated by Jagetia and coworkers as early as 2002.[105] The authors treated human peripheral blood lymphocytes with different concentrations of 1:1 DCM-MET) leaf extract before exposure to 3 Gy γ-radiation, and found protection by reduction of DNA damage as micronuclei that occurred in a concentration-dependent manner. Later on, a study of this extract was conducted to test the radioprotective effect *in vivo*, wherein mice were administered 5, 10, 20, 30, 40, 50, 60 and 80 mg/kg bodyweight of DCM-MET leaf extract. The administration of different doses of DCM-MET leaf extract protected the mice against radiation-induced mortality and sickness in a dose-dependent manner, and the optimum protective dose was found to be 30 mg/kg.[106] Studies were also undertaken to investigate the radioprotective effect of different doses of hydroalcoholic seed extract in mice exposed to a lethal dose (10 Gy), and it was found that the seed extract protected mice against radiation-induced sickness and mortality. Further studies with the optimum dose of 80 mg/kg seed extract resulted in a dose reduction factor of 1.24.[107]

The effect of DCM-MET leaf extract was studied on the intestines of mice treated with 5, 10, 20, 30, 40, 50, 60 and 80 mg/kg bodyweight DCM-MET leaf extract before exposure to different doses of γ-radiation. The mice receiving Jamun leaf extract showed increased villus height and a rise in the number of regenerating crypts, accompanied by a reduced number of goblet and dead cells; this indicated that Jamun extract protected mouse intestine and may have increased the life span of the irradiated mice.[108] The effect of Jamun leaf extract on radiation-induced DNA damage was also studied; mice were administered with 50 mg/kg bodyweight of 1:1 DCM-MET leaf extract before exposure to different doses of γ-radiation. The cells from the irradiated animal spleens were extracted and cultured, and the DNA damage was estimated in cytochalasin B-blocked binucleate splenocytes. The Jamun leaf extract inhibited the production of radiation-induced micronuclei formation and thus protected the mice against radiation-induced DNA damage.[43]

The latest studies carried out in mice to understand the mechanism of action of radioprotection utilized 50 mg/kg bodyweight of DCM-MET Jamun leaf extract before exposure to 0, 0.5, 1, 2, 3 or 4 Gy whole body γ-radiation. The assays for GSH, catalase and SOD revealed that the activities of each were increased significantly in the Jamun leaf extract-treated group, at all exposure doses, compared to the irradiated control group, whereas the induction of lipid peroxidation was reduced in the mouse liver.[109] All these studies have demonstrated that Jamun protects against the radiation-induced mortality, sickness, and intestinal and DNA damage by reducing radiation-induced free radicals and increasing various antioxidants (Fig. 3). Jamun also suppressed inflammatory cytokines, such as NF-κB, iNOS, TNF-α and COX enzymes,[45] which are elevated after exposure to ionizing radiation and may contribute to the radioprotective action (Table 4[43, 105–114]).

Anticancer activity

Cancer is a non-communicable killer disease, which is second only to cardiovascular disease as far as causes of human mortality are

Table 4. The radioprotective and antineoplastic activities of Jamun, *Syzygium cumini* in vivo and in vitro

S. No.	Parts used	Extract type	Activity	Species	References
1.	Leaf	Dichloromethane and methanol (1:1)	Radioprotection	Human Lymphocytes	[105]
				Mice	[43,106,108,109]
2.	Seed	Hydroalcoholic		Mice	[107]
3.	Fruit skin	Crude	Anticancer	HeLa; SiHa	[110]
4.	Fruit pulp	Lyophilized		MCF-7 MDA-MB-231	[111]
5.	Fruit	Methanol Acidified methanol		H460 HCT-116	[114] [113]
6.	Seed	Ethyl acetate Methanol		MCF-7 MCF-7	[112] [112]

concerned. Cancer is treated by surgery, radiotherapy or chemotherapy or a combination of each (or all). In advanced stages, chemotherapy is the only remedy to treat cancer and, hence, it has emerged as one of the most important modalities of cancer treatment.

The majority of cancer treatment drugs (47%) have been derived from natural resources or they are their semisynthetic derivatives.[115] Different parts of Jamun have been investigated for cytotoxic action *in vitro* using a wide array of different cell lines (Fig. 3). The cytotoxic effect of Jamun fruit skin crude extract was studied in HeLa (HPV-18 positive) cells and SiHa (HPV-16 positive) cells by MTT assay, and the crude extract was found to trigger a cytotoxic effect on both the cell types (Table 4). The effect was more pronounced on the HeLa cells than on the SiHa cells, though. Similarly, the effect of 50% methanol extract showed greater apoptosis in HeLa than SiHa cells.[110]

Freeze-dried Jamun fruit pulp extract was found to inhibit cell proliferation and growth of MCF-7 cells and MDA-MB-231 breast cancer cells in a concentration- and time-dependent manner; however, it was less effective in the MCF-10A cells. Yet, the Jamun extract did not induce apoptosis in untransformed MCF-10A breast cancer cells, whereas it was quite effective in triggering apoptotic cell death in both MCF-7 cells and MDA-MB-231 breast cancer cells.[111] The ethyl acetate and methanol extracts of Jamun seeds reduced the cell survival and increased the cytotoxicity in MCF-7 cells in a concentration-dependent manner, and ethyl acetate extract was slightly better than the methanol extract. Almost similar results were reported for DNA fragmentation, an indicator of apoptosis.[112]

Jamun fruit extract has also been reported to induce a cytotoxic effect in a concentration-dependent manner in HCT-116 colon cancer cells. Moreover, the Jamun fruit extract induced apoptosis in HCT-116 and colon cancer stem cells by triggering DNA fragmentation, as determined by TUNEL assay and caspase 3/7 activity.[113] The methanol extract of Jamun fruit has been found to increase the cytotoxicity and suppress cell proliferation in H460 lung cancer cells in a concentration-dependent manner, with an IC50 of 35.2 µg/mL.[114]

Apart from the above-listed activities, Jamun has shown several other properties in different experimental systems, including improvement of memory, antiarthritic activity, anti-nociceptive activity, antigenotoxic effect, central nervous system depressant activity, positive ionotropic effect, antispasmodic activity and many more that are not included in this review.[116–121]

The exact mechanism of action of Jamun in protecting against various disorders is not clearly understood. It seems that Jamun utilizes multiple pathways to exert its conducive effect on different ailments (Fig. 4). Free radical induction has been indicated in several disease processes and the neutralization of excess free radicals by Jamun may be one of the important mechanisms of its action, which is in line with reports of its ability to scavenge different free radicals. Jamun also stimulates the activation of different enzymes, like catalase, glutathione peroxidase, glutathione-s-transferase and SOD, and increases synthesis of GSH, which may have helped in various ways to counter the free radical production, thereby helping to resolve the different diseases.

The reduction of lipid peroxidation may be another reason for its protective effect against several diseases. At the molecular level the presence of Jamun may have inhibited activation of transcription factors, including NF-κB, iNOS, TNF-α and COXs, causing reduced inflammation and protection against various health disorders. Apart from this, Jamun may have also up-regulated the transcription of PPARα and PPARγ, and Nrf2, leading to an increase in the antioxidant status.

Conclusions

The Jamun (*Syzygium cumini*) belonging to family Myrtaceae has been used in traditional medicine for treatment of different ailments, including diabetes. Phytochemical evaluation has shown that Jamun contains alkaloids, anthroquinone glycosides, flavonoids, tannins, saponins, phenols, cardiac glycosides, terpenoids, phytosterols, steroids and amino acids. Several individual components of these phytochemicals have been isolated, as well. Preclinical evaluation has shown that Jamun possesses several medicinal activities, including antioxidant, antibacterial, antifungal, antiallergic, antiinflammatory, antidiabetic antihyperlipidemic, gastroprotective, cardioprotective, hepatoprotective, anticancer, and radioprotective.

Despite the above-listed beneficial and medicinal effects, however, Jamun has some adverse effects in humans. It lowers blood sugar; therefore, it should not be taken at 1 week before, or at a minimum of 2 weeks after, surgery. Jamun should not be taken after drinking of milk and it should be avoided on an empty stomach. Breastfeeding mothers and pregnant women should avoid eating Jamun. Eating excessive amounts of Jamun may cause coughing, sputum accumulation in the lungs, body aches and fever.

The putative mechanisms of action of Jamun may be the scavenging of free radicals, as indicated by increased oxidative stress, elevated activities of catalase, glutathione peroxidase, glutathione-

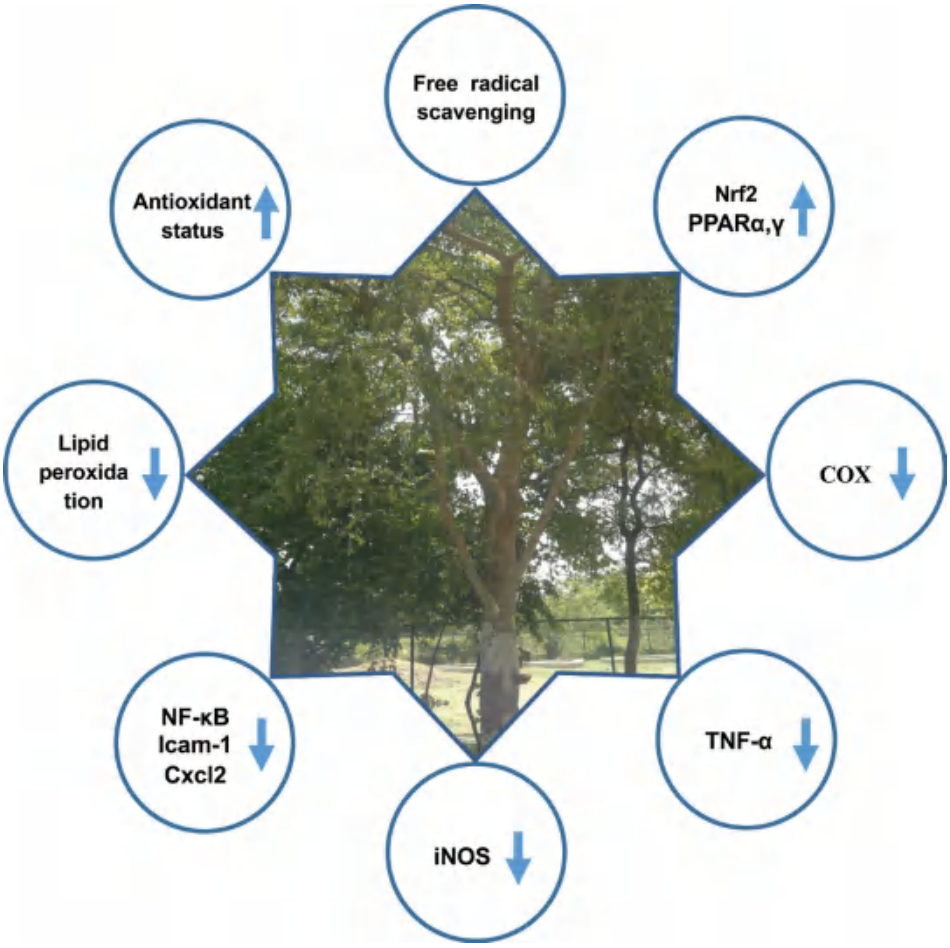

Fig. 4. Plausible mechanism of action of Jamum, *Syzygium cumini.*

s-transferase and SOD, and increased synthesis of GSH coupled with reduced lipid peroxidation. At the molecular level, Jamun may act through its inhibition of transcription of NF-κB, PPARα and γ, COX, iNOS, TNF-α and other inflammatory cytokines, followed by the up-regulation of PPARα and PPARγ, and Nrf2. However, there is need to systematically evaluate the molecular mechanisms of action of Jamun in various study systems.

It is very clear from the collective literature that Jamun has several medicinal properties, and its full potential to treat some of the important disorders of the modern world needs to be further explored. Despite the plethora of studies that indicate its antidiabetic potential, its clinical success seems to be a far cry. Although Jamun fruits are consumed, the toxic implications of Jamun need to be systematically determined in combination with other pharmacologic agents, which will help in realizing its full clinical potential. Finally, the teratogenic effects of Jamun have not been studied, which also indicates the need to thoroughly investigate this aspect in the near future.

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Conflict of interest

The author has no conflict of interest related to this publication.

Author contributions

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