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Dear Esteemed Readers, Authors, and Colleagues,

I hope this letter finds you in good health and high spirits. It is my distinct pleasure to address you as the Editor-in-Chief of Integrative Omics and Applied Biotechnology (IIOAB) Journal, a multidisciplinary scientific journal that has always placed a profound emphasis on nurturing the involvement of young scientists and championing the significance of an interdisciplinary approach.

At Integrative Omics and Applied Biotechnology (IIOAB) Journal, we firmly believe in the transformative power of science and innovation, and we recognize that it is the vigor and enthusiasm of young minds that often drive the most groundbreaking discoveries. We actively encourage students, early-career researchers, and scientists to submit their work and engage in meaningful discourse within the pages of our journal. We take pride in providing a platform for these emerging researchers to share their novel ideas and findings with the broader scientific community.

In today's rapidly evolving scientific landscape, it is increasingly evident that the challenges we face require a collaborative and interdisciplinary approach. The most complex problems demand a diverse set of perspectives and expertise. Integrative Omics and Applied Biotechnology (IIOAB) Journal has consistently promoted and celebrated this multidisciplinary ethos. We believe that by crossing traditional disciplinary boundaries, we can unlock new avenues for discovery, innovation, and progress. This philosophy has been at the heart of our journal's mission, and we remain dedicated to publishing research that exemplifies the power of interdisciplinary collaboration.

Our journal continues to serve as a hub for knowledge exchange, providing a platform for researchers from various fields to come together and share their insights, experiences, and research outcomes. The collaborative spirit within our community is truly inspiring, and I am immensely proud of the role that IIOAB journal plays in fostering such partnerships.

As we move forward, I encourage each and every one of you to continue supporting our mission. Whether you are a seasoned researcher, a young scientist embarking on your career, or a reader with a thirst for knowledge, your involvement in our journal is invaluable. By working together and embracing interdisciplinary perspectives, we can address the most pressing challenges facing humanity, from climate change and public health to technological advancements and social issues.

I would like to extend my gratitude to our authors, reviewers, editorial board members, and readers for their unwavering support. Your dedication is what makes IIOAB Journal the thriving scientific community it is today. Together, we will continue to explore the frontiers of knowledge and pioneer new approaches to solving the world's most complex problems.

Thank you for being a part of our journey, and for your commitment to advancing science through the pages of IIOAB Journal.

Yours sincerely,

Vasco Azevedo

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REVIEW: MOLECULAR CELL BIOLOGY

DECODING CALCIUM SIGNALS IN LIVING CELLS

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ABSTRACT

The regulation of calcium ion concentration outside and inside the cell, together with the movement of calcium ions from one compartment to another, are key factors that determine the fate of a cell: growth, stimulation and death. Calcium is the most ubiquitous second messenger encountered in a plethora of physiological functions from hormonal release and muscular contraction to gene expression. The level of increase in calcium concentration within a cell is finely modulated in terms of the quantity of ions, time and space, thus constituting a complex language that can only be interpreted by specific proteins.

Keywords: *Signalling; Excitation-contraction coupling; Exocytosis; Microdomains; Ion channels*

[I] PHYSIOLOGICAL FUNCTIONS

The homeostasis of calcium ion concentration within a cell is a fine-tuned regulated parameter which presides over many pivotal physiological functions. For example, in the heart, the contraction of cardiac muscle cells is triggered by depolarizing events that induce a small influx of calcium ions, which (through L-type calcium channels or LCCs) in turn leads to the massive release of calcium from intracellular pools (sarcoplasmic reticulum or SR) via calcium release channels known as ryanodine receptors (RyRs). This released calcium binds to the contractile apparatus, which mediates a conformational change and the movement of the myofilaments and therefore a shortening of the cardiomyocyte. This process is referred to as excitation-contraction coupling [1]. In contrast, skeletal muscle cells exhibit a direct mechanical coupling between LCCs and RyRs [2]. In neurones, the cellular localization of calcium channels determines their particular function. At presynaptic sites, calcium channels regulate the release of neurotransmitters by exocytosis. In dendritic spines (that contain the post-synaptic densities where the neurotransmitter receptor complexes are present), calcium signals are responsible for the modulation of learning and memory processes [3]. In particular intracellular inositol-trisphosphate receptors (or $InsP_3Rs$, calcium release channels that are located on the membrane of the endoplasmic reticulum or ER) play a major role as signal detectors and integration centres [4]. In non-excitable cells, $InsP_3Rs$ are the central components of the calcium-induced calcium release (CICR)

phenomenon, but not the only components, since RyRs can also be present and functional such as in the pancreas where RyRs mediate calcium release [5].

Calcium signals can be found in various compartments and organelles of the cell where they display distinct functions. Mitochondria, for instance, serve as calcium buffering systems, and alteration of this pathway leads to pathophysiological states in many varieties of tissues such as in the heart, brain, pancreas and kidney [6]. Moreover, lysosomes also represent calcium stores, and the calcium release from acidic organelles is mediated by nicotinic acid adenine dinucleotide phosphate (NAADP) receptors identified as two-pore channels [7]. It is now accepted that the nucleus itself is involved in the occurrence of calcium signals [8, 9]. These nucleoplasmic calcium signals activate distinct pathways that control gene expression very specifically and independently of cytosolic calcium [10].

Hence, calcium controls many aspects of life at the cellular but also at the organism level, from fertilization of mammalian, sea urchin, fish and frog eggs through development, differentiation and proliferation to the activation of transcription factors and apoptosis [11].

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[II] PATHWAYS INVOLVED IN THE INCREASE OF THE INTRACELLULAR CALCIUM CONCENTRATION

There are 3 main pathways by which a rise in intracellular calcium can occur. They are: i) calcium entry through voltage-gated calcium channels, ii) calcium entry through ligand-gated channels, and iii) calcium release from internal stores.

While the voltage-gated, ligand-gated and store depletion pathways have been studied intensively, the involvement of polymodal (responsive to temperature, light, voltage, ligand, pH and mechanical stimuli) transient receptor potential (or TRP) channels has been the subject of recent investigations [12-14]. These channels are thought to be involved in many physiological functions such as blood pressure, the regulation of mineral absorption/reabsorption, gut motility and airway responsiveness, pain and taste transductions, thermo- and mechano-sensations and cell proliferation/death [15].

In many neuronal and non-neuronal cell types, various molecules (such as peptides, hormones and transmitters) have been shown to specifically bind to their receptors and upon stimulation trigger an influx of calcium *via* activation of phospholipase C (PLC) and adenylate cyclase (AC) pathways, thus exerting cellular functions [16]. Calcium release from stores in mammalian cell types primarily occurs as a result of the activation of metabotropic ligand-binding receptors, which use second messenger signalling cascades to indirectly activate intracellular ion channels.

[III] DIFFERENT TYPES OF CALCIUM SIGNALS

3.1. Calcium sparklets

Optical recordings of localized calcium influx events *via* LCC present in the plasma membrane are called “calcium sparklets”. When a single LCC opens, this leads to calcium influx, resulting in an increase in the total amount of calcium in the cleft between the LCC in the plasma membrane and the opposing cluster of RyRs in the SR membrane. These increases can be recorded as calcium signals named calcium sparklets [17]. These sparklets reveal an unexpected feature of these channels, depending on their activity. In cerebral arterial myocytes, they are of two types, i.e. exhibiting low or high persistent activity, and both are activated by PKC/PKA [18]. Highly elevated PKA-dependent calcium sparklets are observed in cerebral arterial smooth muscle during acute hyperglycemia, vascular dysfunction and diabetes.

3.2. Calcium sparks

The cluster of RyRs that faces the sparklet senses the calcium ions, leading to the synchronous opening of the entire cluster of RyRs and to a highly localised release of calcium out of the SR into the membrane cleft [19]. These elementary signals are thought to sum up to underlie global calcium transients to initiate contraction in the heart. Calcium sparks are usually characterised by the amplitude, the duration, the rise time, the spatial spread of the Ca^{2+} signal, and by the frequency (of occurrence of events) of individual spark sites or the frequency of all the sparks within one cell [20]. In smooth muscles, sparks can occur spontaneously or after activation by many factors such as caffeine, calcium entry via L-type calcium channels, increase in SR calcium content and stretch (see for details: [21]). It should be noted that calcium sparks are the only small physiologically relevant elementary calcium release events in cellular calcium signalling and that the corresponding homogeneous calcium release remains unexplained. However, calcium sparks have been shown to be a major signalling pathway for excitation-contraction coupling.

3.3. Calcium quarks

There is yet another phenomenon in which highly localized SR calcium release in both skeletal and cardiac muscles may be triggered under certain conditions, i.e. calcium quarks [22]. By using two-photon photolytic activation of localized calcium release in cardiac myocytes, it has been shown that a single calcium spark actually consists of the summation of unitary calcium events characterised by tiny amplitudes, a very short lifetime and tight spatial confinement. Hence, each individual RyR could give rise to calcium releases in the dyadic cleft called calcium quarks [22]. However, this phenomenon remains unclear (see also: [23]).

3.4. Calcium puffs

Local intracellular calcium signals caused by the opening of clustered InsP3 receptors in the ER or SR membrane are known as calcium puffs [8, 24]. Calcium puffs display a longer lifetime in comparison to calcium sparks and a wider spread. Each calcium puff would be composed of elementary events named calcium blips [25]. Calcium puff events have been characterised in neurones [26] and in smooth muscle cells [27].

3.5. Calcium marks, scraps and blinks

Microdomain miniature calcium transients in single mitochondria are termed "marks" and SR luminal calcium depletion transients are termed "scraps".

In the cardiac cell line H9C2, it has been shown that subsequent to calcium spark events, rapid increases in calcium in the neighbouring mitochondria can occur [28]. It was proposed that calcium marks are triggered by RyRs through a process involving the travel of calcium from spark sites into the mitochondria.

Attempts were made to monitor calcium movements within the SR lumen, which led to the observation of local depletions of the intracellular calcium concentration (“scraps”), mirrors of the calcium transients recorded in the cytosol [29]. Likewise, rapid and substantial depletions of calcium from the nanometer-sized luminal stores in heart cells have been shown and called "calcium blinks", blinks meaning the confined SR depletions from single calcium sparks [30, 31]. Therefore, the visualization of these local store calcium signals can be an important key to understand cardiac physiopathology.

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3.6. Calcium waves

Calcium waves are global increases of the intracellular calcium concentration from the SR or ER that propagate throughout the cell, sustained by the phenomenon of CICR. These events have a longer lifetime and a larger amplitude than the elementary events previously described and can appear in several forms (spirals, U- and V-shapes and circular waves). The analysis of their kinetics gives indications about the activity of proteins involved in buffering systems such as SR/ER calcium ATPase (SERCA) pumps [32].

All these different types of calcium signals modulate various physiological functions specific to particular cell types.

[IV] CALCIUM HANDLING MECHANISMS

The common method to detect changes in the intracellular calcium concentration is to use fluorescent calcium probes (Fura-2, Fluo-3, Fluo-4, Indo-1, Calcium Green, Rhod-2, or Oregon Green 488 BAPTA-1, this list being obviously not exhaustive) and to monitor the corresponding signals over time. However, one has to bear in mind that such techniques only enable the investigator to detect free calcium ions, i.e. calcium which is not bound to proteins, whereas most of the calcium ions in the cell are bound. The role of such calcium-binding proteins is not only important for creating a buffering system, but also for interpreting calcium signals and triggering molecular responses accordingly. Indeed, it seems difficult to relate the individual, short and sometimes highly localized increases of calcium concentration to other signalling processes such as phosphorylation events, which one assumes would be more global and of longer onset/duration.

Recently, a nice study has provided the beginning of an answer to that question. The authors expressed the calcium-dependent protein kinase C (PKC) isoform alpha fused to various fluorescent proteins in HEK293 and COS1 cells. By confocal imaging, they could monitor the PKC translocation events from the cytosol to the plasma membrane. They successfully demonstrated for the first time the relationship between global and local calcium signals on the one hand, and global and local PKC translocation events on the other hand. It should be noted that they evidenced that calcium signals and PKC translocations were concomitant [33].

Therefore, PKCalpha is a cellular "calcium sensor" following the spatio-temporal characteristics of each calcium signal. Whether these PKC translocation events are associated with phosphorylation events is extremely likely but still needs to be investigated.

Calcium fluctuations are sensed by a wide array of "calcium sensors" which have one common feature: they are all endowed with calcium-binding sites, such as in calcium-sensitive enzymes, with different affinities for calcium, and the binding of the latter modifies their activity by changing their conformation, which therefore triggers or discontinues diverse biochemical processes, which in turn control various cellular reactions [34]. The duration and kinetics of intracellular calcium fluctuations control the timing of calcium-binding/unbinding to the "calcium sensors", thus forming the basis for the temporal coding of calcium signalling events [35]. There are a few main intracellular compartments intimately involved in creating, encoding and decoding calcium signals, which are the cytoplasm, the Golgi complex, lysosomes, secretory granules, the nucleus, the endoplasmic reticulum and the mitochondria [36]. Furthermore, the formation of intracellular free calcium fluctuations is controlled by calcium channels and transporters, which, being activated by physiological stimulations, precisely regulate the calcium ion concentration [35].

[V] CALCIUM CLEARANCE MECHANISMS

Calcium clearance mechanisms are important to maintain calcium homeostasis within a range of concentrations that allow biochemical reactions to take place but are not toxic to cellular functions [37]. Indeed calcium removal (or calcium clearance) is even decisive for the fate of the cell, which includes the "cell death" process when the cell has lost the capacity to extrude or reuptake the extra amount of calcium [38, 39]. The mechanisms involved in intracellular calcium buffering and restoration to basal levels include calcium pumps in both intracellular compartments and the plasma membrane, mitochondrial calcium uptake, plasma membrane sodium/calcium exchange and calcium-binding proteins in the cytosol **[Figure-1]**. Depending on the cell type, the mechanisms of calcium homeostasis and the implications of each of the clearance systems mentioned above will be different.

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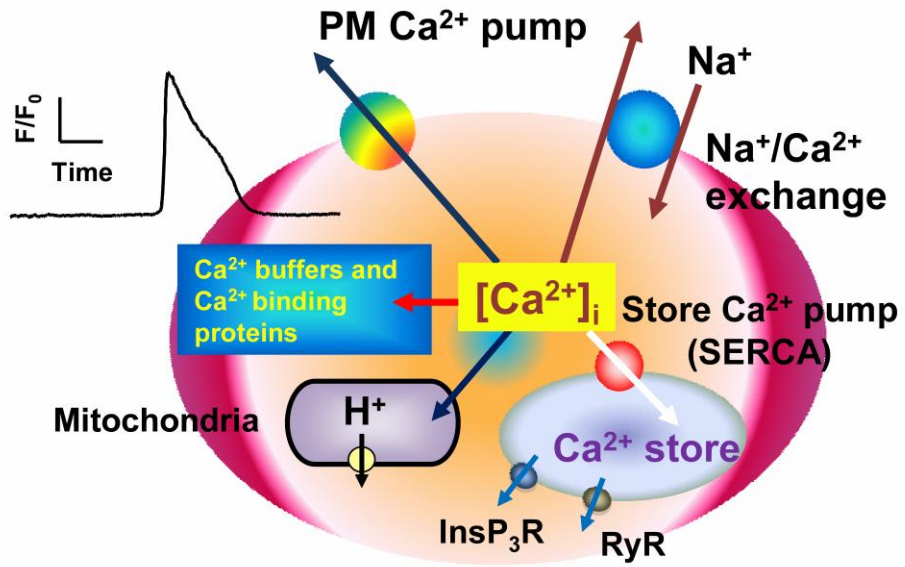

Fig: 1. Schematic representation of possible calcium clearance mechanisms in neuronal, muscle and non-excitable cells: Arrows suggest that increased cytosolic calcium can be extruded *via* plasma membrane (PM) Ca^{2+} pump, Na^{+}/ Ca^{2+} exchange, stored by mitochondria, intracellular compartments (*via* sarco/endoplasmic reticulum Ca^{2+} pump, SERCA), Ca^{2+} buffers and Ca^{2+} binding proteins. Abbreviations: ryanodine receptor (RyR), inositol-trisphosphate receptor ($InsP_3R$), intracellular calcium concentration ($[Ca^{2+}]_i$) and normalized change of the calcium-dependent fluorescence (F/F_0). A representative $[Ca^{2+}]_i$ transient (recorded with a fluorescent calcium indicator) is depicted in the top left corner of the cartoon.

[VI] CONCLUSION

Calcium is the key to a wide variety of cellular processes. Monitoring and deciphering calcium signals can be a useful tool to predict how cells are likely to behave. Besides the understanding of essential physiological phenomena, the exploration of the readout of calcium events by calcium detectors (fluorescent or endogenous calcium-binding proteins) could have applications in detecting pathophysiological remodelling, arrhythmic cardiac patterns and neurological disorders such as Alzheimer's disease [40]. Once typical calcium signal patterns can be identified and defined, this will be useful in developing drug screening strategies and drug therapies.

FINANCIAL DISCLOSURE

The authors state that they have no conflict of interest pertaining to this manuscript.

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RESEARCH: NUTRACEUTICALS

HPLC ANALYSIS OF BIOACTIVE COMPOUNDS IN TEN DIFFERENT WILD TYPE UNDER-UTILIZED LEGUME GRAINS

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ABSTRACT

In recent years, many food industries have been initiated the formulation of nutraceutical/functional foods by incorporating the bioactive ingredients for the prevention/treatment of certain chronic diseases. In this connection, certain promising wild type under-utilized legume grains received more attention, since they are naturally a rich source of L-Dopa (precursor of dopamine) and certain bioactive compounds including phenolics, tannins and phytic acid. In the present study, seed materials of certain promising wild type under-utilized food legume grains such as* Abrus precatorius *L.,* Acacia leucopholea *Willd,* Bauhinia varigata *L.,* Canavalia gladiata *(Jacq.) DC.,* Cassia floribunda *Cav.,* Entada scandens *Benth.,* Indigofera linifolia *(L.f.) Retz.,* Mucuna monosperma *DC. Ex Wight.,* Sesbania bispinosa *(Jacq.) Wight. and* Tamarindus indica *L., collected from Eastern and Western Ghats of South India, were investigated for certain bioactive compounds through HPLC technique. All the analysed samples were found to constitute a viable source of total free phenolics (4.23 – 8.75 g/100 g DM), tannins (1.04 – 5.41 g /100 g DM), L-Dopa (1.17 – 5.34 g/100 g DM) and phytic acid (0.96 – 2.74 g/100 g DM) and also the newly developed HPLC procedures were proven to be sensitive enough to detect these bioactive compounds even at tracer level. Further, such wild type legume grains could be recommended as a natural source of bioactive compounds in the dietary management of certain chronic diseases such as Parkinsonism, diabetes, obesity, cardiovascular diseases, cancer etc.

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Keywords: *Wild type legume grains; bioactive compounds; total free phenolics; tannins; L-Dopa; phytic acid*

[I] INTRODUCTION

Legume grains have been playing a key role in the traditional diets of human beings throughout the world. They are excellent source of protein, dietary fibre, starch, micronutrients and bioactive compounds with low level of fat. The total per capita consumption of legume grains has been increased markedly over the past two decades in US, due to increased attention to beans being classified as functional foods [1]. Accumulation of chemical, biochemical, clinical and epidemiological evidences indicating a positive correlation between the consumption of legume seeds and decreasing incidence of several chronic diseases such as cancer, cardiovascular diseases, obesity and diabetes [2]. Such obvious health benefits of legume seeds are attributed to presence of certain bioactive compounds such as phenolic acids, flavonoids and tannins [3]. Therefore, at present the studies on bioactive compounds, which are responsible for health promoting/disease preventing effect, are being increased in addition to the evaluation of nutritive profiles of legume grains.

Actually in olden days, the bioactive compounds like total free phenolics, tannins, L-Dopa and phytic acid were considered as antinutritional substances and their presence in food/feed-stuffs was reported to be undesirable from the nutritional point of view. But, now-a-days, the health beneficial role of such bioactive compounds has been explored by a large number of research studies. Particularly, these bioactive compounds were demonstrated to possess many favourable medicinal properties, including potential antioxidant activity [3,4,5]. As a consequence of health beneficial effects, presence of such bioactive compounds in the diet has been viewed in a positive light in recent years by both scientists and consumers and resulted in a push to procure foods with specific health benefits such as functional foods.

Apart from common legume seeds, the earlier research efforts revealed the nutritive potential of certain promising under-utilized/wild legume seeds, including the pulses of tribal

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utility. Among the various under-utilized legumes, the seed materials of *Abrus precatorius* L., *Acacia leucopholea* Willd, *Bauhinia varigata* L., *Canavalia gladiata* (Jacq.) DC., *Cassia floribunda* Cav., *Entada scandens* Benth., *Indigofera linifolia* (L.f.) Retz., *Mucuna monosperma* DC. Ex Wight., *Sesbania bispinosa* (Jacq.) Wight. and *Tamarindus indica* L. merit a wider use as a food legume. Their distribution, agronomic traits, nutritional value, mode of consumption was described in detail by Janardhanan *et al*. [6]. In South India, these wild type legume grains are being traditionally consumed by certain ethnic groups, particularly the Kanikkar, Lambadi, Uraali and Dravidian tribes living in Tamilnadu, Kerala, Karnataka and Andhrapradesh States.

Even though, few reports are available regarding the nutritional value of the above-mentioned under-utilized legume grains, information on bioactive compounds are very meagre and also no standard HPLC methods were reported for the analysis of total free phenolics, tannins, L-Dopa and phytic acid. Hence, a very sensitive and reliable HPLC technique should be developed in order to detect these compounds, before using them in the dietary management trials of various oxidative-stress related diseases. So that, we can easily measure these compounds in the biological samples like blood plasma, urine, etc., more accurately within short period of time. In the present study, an attempt has been made to develop suitable HPLC methodologies to analyze the levels of bioactive compounds of ten different under-utilized legume seeds collected from Eastern and Western Ghats of South India.

[II] MATERIALS AND METHODS

2.1. Chemicals

Poly-vinyl-polypyrrolidone, (+)-catechin hydrate, vanillin, tannic acid, L-Dopa, phytic acid were procured from Sigma-Aldrich Chemicals, USA; Sephadex LH-20 was obtained from Pharmacia Fine Chemicals, Sweden; Anion exchange resin was purchased from Bio-Rad, USA, and all other chemicals and HPLC grade solvents were received from Merck, Darmstadt, Germany.

2.2. Collection of seed samples

The details on collection of seed materials of wild type legume grains from different agro-climatic locations of Eastern and Western Ghats of South India were given in Table 1. After removing the immature and damaged seeds, the mature seeds were dried under shaded condition for two days. All the samples were freezed at -80°C and freeze-dried for 48 h. Then the samples were first cracked with the help of a wooden hammer into small pieces and subsequently powdered in a seed mill (Siemens, Germany) to 1 mm particle size, freeze-dried for 24 h and stored at 9°C until further use.

2.3. Total free phenolics

The total free phenolics were extracted from seed samples by taking 1 g of defatted seed flour sequentially with 10 ml of 100%, 80% and 50% methanol and 70% acetone acidified with 1% conc. HCl in an ultra-sonic bath (Bandelin Sonorex, RK – 514 H, Berlin, Germany) for 30 min. After centrifugation, all the supernatants were collected and made up to a known volume. The extract was treated with 500 mg of poly-vinyl-polypyrrolidone at 0°C for 30 min. Then the contents were purified by using a Solid Phase Catridge (SPC) (Strata-x-33 um polymeric sorbent, L100-1105, 200 mg/6 ml sample, 8B-S100-FCH-S from Phenomenex, USA). Then the solvents were evaporated in a rotary-vacuum evaporator (Büchi Rotavapor – R, CH-9230, Switzerland) at 40°C and 25 mbar pressure and dried in lyophilier (Virtis Freeze-mobile – 25 EL, New York). The contents were re-dissolved in 1 ml of solvent (Methanol:water:formic acid, 47.5:47.5:5%) and transferred to a HPLC vial. The HPLC apparatus consists of Varian Prostar-210 High pressure gradient pump, Waters-2487 Dual absorption detector, Triathlon autosampler (Spark Holland, The Netherlands), operated by Galaxy Chromatography Data Systems (GCDS), version-1.9.3.2 from Varian. The column consists of Reprosil-Pur 120 C18 AQ 5 µm (250 x 4.6 mm size) column from Trentec, 71273 Rutesheim, Germany, which is maintained at 40°C. Solvent A (water and formic acid, 95:5%, v/v) and solvent B (water, acetonitril and formic acid, 85:10:5%, v/v/v) were used as mobile phase with the gradient of 20% B at 0 min, 32% B at 6 min, 63% B at 6.01 min, 84% B at 11 min, 100% B at 11.01 min100% B at 15 min, 20% B at 15.01 min and 20% B at 20 min. Flow rate of 1 ml/min was used and the signals were detected at 280 nm. Based on the standard curve prepared with (+)-Catechin hydrate (20-100 µg), the amount of total free phenolics in the extract was calculated and expressed in g/100 g seed flour on dry matter basis.

Name of the wild type legume grain	Colour of the seed coat	Place of collection
Abrus precatorius	Red & black	Guruvayur, Kerala State
Acacia leucopholea	Black	Bhavanisagar, Tamilnadu State
Bauhinia varigata	Light brown	Mysore, Karnataka State
Canavalia gladiata	Light brown	Guntur, Andhrapradesh State
Cassia floribunda	Reddish brown	Kodivery, Tamilnadu State
Entada scandens	Dark brown	Tirunelveli, Tamilnadu State
Indigofera linifolia	Grey	Kollegal, Karnataka State
Mucuna monosperma	Light brown	Thiruvalla, Kerala State
Sesbania bispinosa	Dark green	Kadambur, Tamilnadu State
Tamarindus indica	Dark brown	Kanyakumari, Tamilnadu State

Table: 1. Data on collection of seed materials of certain wild type legumes collected from different agro-ecological locations of South India.

2.4. Tannins

The tannins were extracted from the seed materials by taking 1 g of defatted seed flour sequentially with 100%, 90%, 80% and 70% acetone solutions acidified with 1% conc. HCl. After centrifugation, all the supernatants were pooled together and made up to a known volume with acetone. Then the extract was purified by using Sephadex LH-20 column chromatography (96 x 1.6 cm) with acetone:water (50:50, v/v) as a solvent [2]. After collecting 20 fractions (5 ml each), the active fractions were identified and pooled together, evaporated and lyophilized as described above and used for analysis. For HPLC/PDA analysis, 20 µl was injected into a Varian HPLC (Pro Star

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210) equipped with a Shimadzu PDA (PD-M20A) applying the following chromatographic conditions: Reprosil-Pur 120 C18 AQ column (5µm, 250 x 4.6 mm) at 40°C, and solvent A of aqueous formic acid (5% v/v) and solvent B containing formic acid, destilled water and acetonitril (5:10:85, v/v/v). The gradient program was as follows: 5% B to 26% B (12.38 min), 26% B to 100% B (17.22 min), 100% B isocratic (10 min) with a flow-rate of 1 ml/min and a total run time of 40 min. and the signals were detected at 280 nm. The standard curve was prepared by taking different concentrations of tannic acid and the level of tannins was calculated.

2.5. L-Dopa

Finely ground seed flour (1 g) was treated with 10 ml of petroleum ether and kept in an ultra-sonic bath for 30 min. Then the defatted pellet was extracted with 10 ml of 0.1 N HCl. The contents were vortexed for 10 min at room temperature (25°C) and kept in an ultra-sonic bath for 30 min under ice bath condition and subsequently it was kept on a magnetic stirrer for 1 h at room temperature. The supernatant was collected by centrifugation (13,000 x g, 15 min) and the extraction procedure was repeated twice and all the supernatants were pooled and diluted to a final known volume and used for further analysis. Then the extract was evaporated and lyophilized and re-dissolved in water the ratio of 5 mg extract/ml. The same HPLC system described above was used with the eluting solution (Solvent A: water, methanol and phosphoric acid in the ratio of 975.5:19.5:1, v/v/v, pH 2.0), and washing solution (Solvent B: 70% methanol). The gradient used was: start with 100% (A) and 0% (B) up to 12 min, next 5 min solvent (B) increase from 0 to 100%, decrease B to 0% in the next 5 min, and then the column is washed with solvent- A alone in the next 15 min to adjust the column to the starting conditions. Isocratic elution was carried out and the separation was performed at room temperature (25°C) at the flow rate of 1 ml/min and the signals were detected at 282 nm. The L-Dopa content was calculated based on the standard curve prepared with synthetic L-Dopa.

2.6. Phytic acid

The phytic acid was extracted from raw and differentially processed seed samples by taking 1 mg of defatted seed flour with 10 ml of 2.4% HCl and incubated for 10 min in ultra-sonic bath. Then the contents were centrifuged at 13,000 x g for 5 min and the supernatant was collected. Similarly, the residue was re-extracted twice and all the supernatants were pooled together and made up to a known volume with distilled water. The extract was purified by using an anionic-exchange column chromatography (0.7 cm x 15 cm) containing 0.5 g of anion-exchange resin (100–200 mesh, chloride form; AG1-X4, Bio-Rad Co., CA, USA). The phytic acid was eluted with 2 M HCl and used for quantification. The aliquot was analyzed on a Merck-Hitachi HPLC (LaChrom) equipped with a column oven (set at 40°C), fluorescence detector (L-7480) and Clarity chromatographic station (DA-C50, DataApex Ltd, Praha). The separation was achieved on a 5 µm analytical column (Grom-Sil 120 ODS-4 HE, 125 x 4 mm, Grom, Rottenburg-Hailfingen, Germany) using a mobile phase consisting of methanol (27.5% v/v) and phosphate buffer (pH 7.0) at a flow rate of 0.8 ml/min. Phytic acid was detected by excitation/emission set at 367/435 nm.

2.7. Statistical analysis

All the data were analyzed and expressed as means ± standard deviation of five separate determinations (n = 5). The statistical analysis was carried out by using SPSS for Windows (SPSS Inc., Chicago, IL, version 11.0). Values of analyzed compounds were found to be normal distributed by using Kolmogorov-Smirnov-test. Means of the groups were compared by one-way ANOVA and Dunnett post-hoc test using the raw seeds as a control. Two-tailed P values < 0.05 were considered statistically significant.

[III] RESULTS AND DISCUSSION

3.1. Total free phenolics

The phenolic compounds constitute one of the most numerous and ubiquitously distributed group of plant secondary metabolites, which are ranged from simple molecules (eg. phenolic acids, phenyl-propanoids and flavonoids) to highly polymerized compounds (eg. lignins and melanins). Now-a-days, the phenolic compounds are demonstrated to prevent the development of many chronic diseases such as atherosclerosis, diabetes, cancer *etc*. Such protective effect of phenolics might be associated with their powerful antioxidant and free radical scavenging properties [7]. The seed coat of legume grains are reported to contain numerous types of phenolics, which playing an important protective role against oxidative damage in consumer's body [2].

The total free phenolics content of raw seed materials of different wild legume grains were found to range between 4.23 and 8.75 g/100 g seed flour DM **[Figure-1]**. These values are higher when compared to the previous reports on broad bean (2.39 g/100 g DM); pea (2.26 – 3.48 g/100 g DM); white bean (1.08 g/100 g DM); black bean (4.40 g/100 g DM) and common bean (1.88 – 2.53 g/100 g DM), but comparable with that of faba bean (5.59 g/100 g DM); Adzuki bean (8.97 g/100 g DM); red bean (5.54 – 9.36 g/100 g DM); red lentil (5.80 g/100 g DM); green lentil (6.76 g/100 g DM) and brown bean (9.14 g/100 g DM) [8].

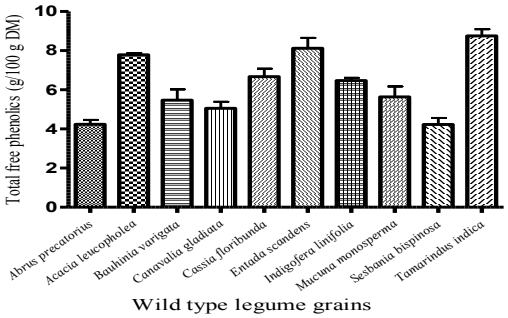

Fig: 1. Total free phenolics content of ten different wild type legume grains

In general, the total free phenolics content of presently investigated under-utilized legume grains were appears to be higher when compared to the literature [6]. This might be due to the repeated extraction of phenolic compounds by using both methanol and acetone as solvents as well as sensitivity of HPLC method **[Figure-2]**. Recovery of phenolic compounds from legume grains is mainly depends upon the type of solvent used and the duration of extraction. Acetone and methanol extracts of seed samples exhibited higher phenolic yield when compared to either methanol or acetone used alone [9].

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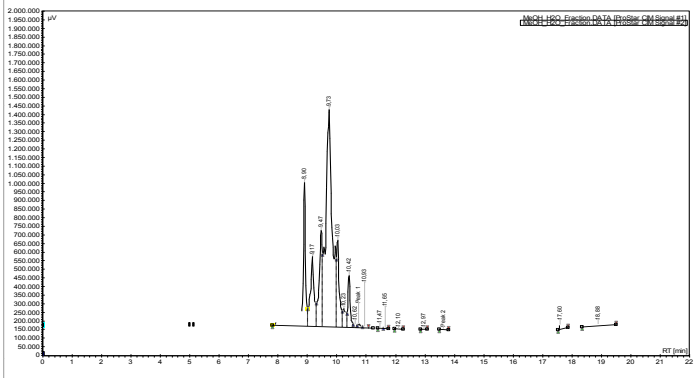

Fig: 2. HPLC analysis of total free phenolics in wild type legume grains.

The seed samples of *Tamarindus indica* (8.75 g/100 g DM) registered significantly ($p < 0.05$) higher level of total free phenolics, which is followed by *Entada scandens* (8.12 g/100 g DM) and *Acacia leucopholea* (7.79 g/100 g DM) **[Figure-3]**. It is interesting to notice that the seed coat colour of these seed materials is dark brown/black. Relationships between seed coat colour and phenolics level are still controversial. While Barampama and Simard [10] found a positive relationship between the seed coat colour and phenolic content, Guzman-Maldonado *et al*. [11] did not find any relationship. However, there are some reports available with high correlation between cultivar lines and phenolic content [12]. In addition to seed coat colour, it is well documented that the quantity of phenolic compounds in seed samples is influenced by soil, environmental conditions, genotype (cultivar/variety), agronomic practices (irrigation, fertilization and pest management), maturity level at harvest and post-harvest storage. For instance, low temperature during the onset and duration of seed fill were shown to increase the isoflavone content by several folds in soybean [13]. Since these under-utilized legumes grow wildly in adverse environmental conditions such as drought, poor soil *etc*., a high phenolic content contributes to the resistant function.

Although the dietary intake of phenolics varies considerably among the geographical regions, it is estimated that the daily intake of total free phenolics was ranged from 20 mg to 1 g, which is higher than the intake of vitamin E. Hence, in recent years, food technologists are keen to harness the nutritional benefits of phenolics, namely its antioxidant or free radical scavenging, food preservative, antimicrobial, anti-mutagenic, therapeutic and pharmaceutical properties.

3.2. Tannins

Beside simple phenolics mainly found in cellular vacuoles, some polymerized form of phenolics with varying degree of solubility such as tannins are also noticed in legume seeds. Tannins are defined as a unique group of phenolic metabolites of relatively high molecular weight. Concerning chemical structure, they can be divided into four groups: condensed tannins, hydrolysable tannins, phlorotannins and complex tannins [14]. Tannins possess ideal structural chemistry for better free radical scavenging activity and hence, they exhibit more effective antioxidant activity under *in vitro* conditions than tocopherols and ascorbic acid [15]. The free radical scavenging power of tannins is closely connected with their spatial confirmation and degree of polymerization. Further, both the hydrolysable and condensed tannins are demonstrated to possess more effective and greater antioxidant activity than simple phenolics.

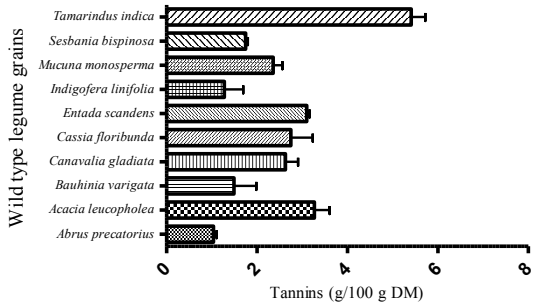

Fig: 3. Tannins content of ten different wild type legume grains.

The tannins content of raw under-utilized legume grains were found to falls between 1.04 and 5.41 g/100 g DM **[Figure-3]**. These values are found to be higher when compared to previous reports on green pea (0.003 – 0.17 g/100 g DM); yellow pea (0.15 g/100 g DM); chickpea (0.18 g/100 g DM); lentil (0.012 – 0.88 g/100 g DM); red kidney bean (0.012 – 0.55 g/100 g DM); black bean (0.04 – 0.67 g/100 g DM) and common bean (0.02 – 0.13 g/100 g DM) [8]. Such a high level of tannins in wild legume seeds when compared to the literature [6] might be due to the type of solvent used for extraction as well as accuracy of the presently developed HPLC technique **[Figure-4]**. Similarly, Chavan *et al*. [16] and Troszyska *et al*. [2] reported the maximization of extraction of tannins from beach pea and yellow pea seed coats, respectively, when acetone was used as a solvent compared to methanol.

It is noticeable that, the seed samples with dark brown coloured seed coat like *Tamarindus indica* (5.41 g/100 g DM) and *Entada scandens* (3.10 g/100 g DM) as well as black coloured seed coat *Acacia leucopholea* (3.27 g/100 g DM) were registered significantly ($p < 0.05$) higher level of tannins than the other seeds.

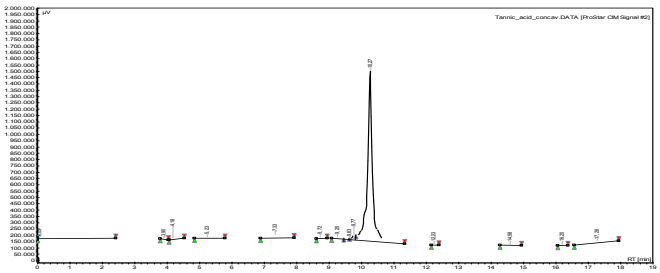

Fig: 4. HPLC analysis of tannin in wild type legume grains

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It is postulated that high level of condensed tannins or proanthocyanidin are seen in dark coloured beans than in yellow or white coloured beans. Since, the level of phenolics was relatively low in pale coloured seeds; it is possible to assume that the major phenolics in dark coloured coated seeds could be proanthocyanidins. Recent studies have demonstrated a quantitative pattern of heredity for tannins content and that tannins level is also associated with seed coat colour inheritance.

Several factors, such as plant type, cultivar, age of the plant or plant parts, stage of development and environmental conditions were reported to govern the tannins content in legume grains. Presence of high content of tannins in the presently studied wild legume seeds might be due to the metabolism of polyphenolic compounds or polymerization of existing phenolic compounds during development or maturation [16]. According to Serrano *et al*. [14], the mean daily intake of condensed tannins among US population (>2 year old) was estimated to be 53.6 mg/person/day, whereas 450 mg/person/day in the Spanish diet. There are a lot of epidemiological data, which suggested that tannins intake may prevent the onset of many chronic diseases. The positive biological effects including antioxidant, anticarcinogenic, anti-mutagenic, antimicrobial, antiviral and anti-diabetic properties of tannins have been extensively studied.

3.3. L-Dopa

L-Dopa (L-3,4-Dihxdroxyphenylalanine) is a non-protein phenolic amino acid, mainly used in the treatment of Parkinson's disease, since it is the precursor of dopamine. L-Dopa has also been investigated as a dietary supplement to manage hypertension, renal failure and liver cirrhosis. Further, the protective effects of L-Dopa on small bowel injury, ulcer, gastro-intestinal diseases, diabetes as well as antioxidant stress were scientifically proved by earlier studies [17]. The seed materials of wild legume grains, especially the *Mucuna* sp. was reported to contain appreciable level of L-Dopa [18].

The raw seed materials of different wild legume grains of the present study recorded the L-Dopa content of 1.17 - 5.34 g/100 g DM **[Figure-5]**. These values are found to be comparable with that of certain under-utilized legumes such as *Cassia floribunda* (1.57 g/100 g DM); *C. obtusifolia* (1.34 g/100 g DM); *Canavalia ensiformis* (2.64 g/100 g DM) and *C. gladiata* (2.83 g/100 g DM) [19], but, lower than that of *Mucuna cochichinensis* (6.11 g/100 g DM) and *M. veracruz* (7.12 g/100 g DM) [20]. The presently developed HPLC method is found to be more sensitive **[Figure-6]**, but has a drawback that the compound should be analyzed within 8 h of extraction to avoid the oxidation of L-Dopa.

The L-Dopa content varies considerably at significant level ($p < 0.05$) among the wild legumes of the present investigation. The seed samples of *Mucuna monosperma* recorded the maximum level of L-Dopa content (5.34 g/100 g DM), while the low level was observed in *Tamarindus indica*.

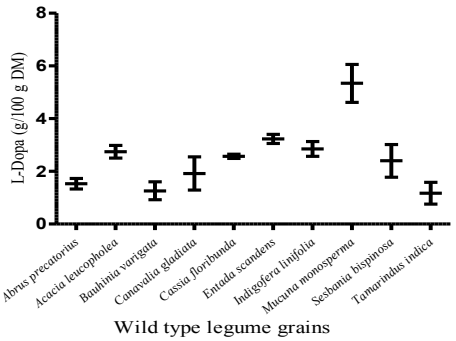

Fig: 5. L-Dopa content of ten different wild type legume grains.

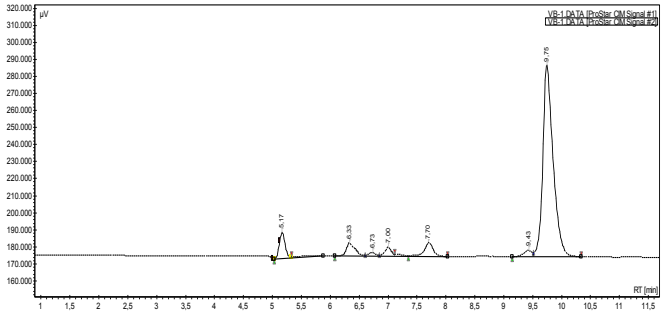

Fig: 6. HPLC analysis of L- Dopa in wild type legume grains

In general, the *Mucuna* species is naturally a potential source of L-Dopa and commercially used for the extraction of this compound for the treatment of Parkinsonism. Such a wide variability in L-Dopa content among wild legumes could be cause by both environmental effect and genetic nature. For instance, presence of more L-Dopa was noticed in the *Mucuna* plants growing near the equator (within 10°) than the plants cultivated far away from equatorial regions in earlier investigations. Further, the L-Dopa synthesis is reported to be high in plants growing at low latitudes, near the equator [18]. It was also hypothesized that variation in the intensity of light and backscattered ultraviolet radiation, both generally more near the equator, may be among the factors explaining why the L-Dopa content was found to be high in plants growing at low latitudes.

3.4. Phytic acid

Phytic acid (myo-inositol hexaphosphate) is widely found in cereals, nuts, legumes, oil seeds, pollen and spores, constituting about 1–5% and generally the legume seeds are regarded as the major source of dietary phytate [4]. In recent years, the phytic acid is considered as an antioxidant, anti-carcinogenic, hypoglycemic and hypolipidemic agent, in addition to the fact that a high phytate diet can be effectively used in the treatment of hyper-calciuria and kidney stones in human beings [21]. The wild type legume seeds were found to contain 0.96 - 2.74 g/100 g DM of phytic acid **[Figure-7]**.

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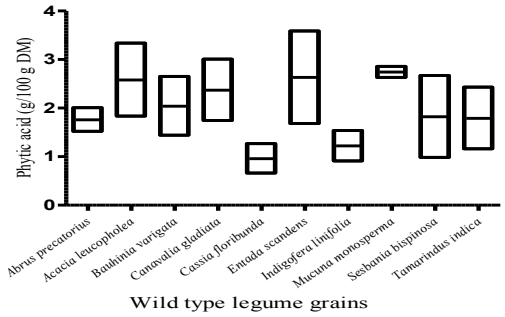

Fig: 7. Phytic acid content of ten different wild type legume grains.

These values are comparable with that of an earlier report on *Phaseolus vulgaris* (0.61 – 2.38 g/100 g DM); *Vicia faba* (0.51 – 1.77 g/100 g DM); *Pisum sativum* (0.22 – 1.22 g/100 g DM); *Vigna unguiculata* (0.37 – 2.90 g/100 g DM); *Cicer arietinum* (0.28 – 1.60 g/100 g DM) and *Lens culinaris* (0.27 – 1.51 g/100 g DM) [21].

The HPLC chromatogram revealed the presence of two different fractions (tetra- and penta-phosphate myoinositol) of phytic acid **[Figure-8]**. Considerable level of variation on the phytic acid content of presently investigated under-utilized legume grains might be attributed to both genetic and environmental conditions. In general, the cultivar, which contains appreciably high amount of protein is observed to be The estimated daily phytic acid intake of human population was about 750 mg in U.S.A.; 600 - 800 mg/day in U.K.; 393 mg/day among Canadian children; 2000 - 2200 mg/day in Nigeria; 1890 and 569 mg/day in Malawi and New Guinea, respectively and 1487 mg/day in East India [21]. But, historically it has been considered as an antinutrient and postulated to impede the bioavailability of minerals. Nevertheless, the research studies conducted by Grases *et al*. [23] showed that there is no negative effect on the mineral balance and element bioavailability due to the oral administration of phytic acid, even in the second generation rats.

[IV] CONCLUSION

The HPLC techniques developed to analyze the bioactive compounds of under-utilized legume samples were found to be more appropriate and sensitive enough to detect the total free phenolics, tannins, L-Dopa and phytic acid even at tracer levels. Ten different wild type unconventional legume seeds collected from various agro-ecological regions of South India were found to constitute rich source of bioactive compounds. Among the under-utilized legume grains, significantly high level of total free phenolics and tannins were noticed in *Tamarindus indica*, *Entada scandens* and *Acacia leucopholea* seeds, while *Mucuna monosperma* registered maximum level of L-Dopa and phytic acid. Hence, presently studied wild type legume seeds could be recommended in order to increase the dietary intake of health beneficial bioactive compounds. Such associated with high phytic acid content. Hence, as the protein content increases, the phytate level is also found to increase in the seed samples. Al-Numair *et al*. [22] reported that the amount of phytic acid is always exceeds than that of phosphorus for all the legume cultivars, which indicates that the ratio would be more than 100%. Generally, in legume seeds, the phytic acid level is positively correlated with total phosphorous, correlation coefficients being greater than 0.90. Factors that affect the total phosphorous content, such as soil, available phosphorous and fertilizer can also influence the phytic acid concentration.

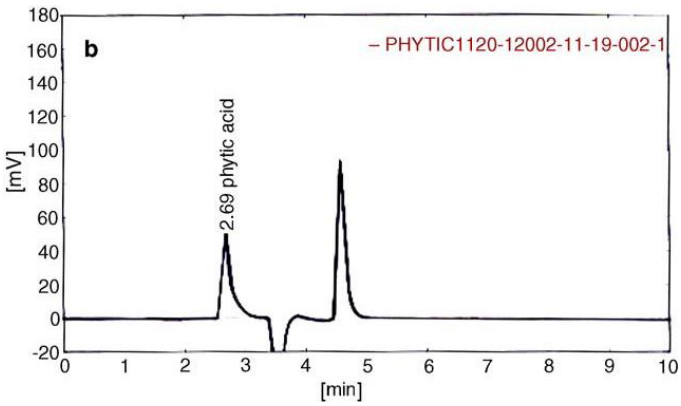

Fig. 8. HPLC analysis of phytic acid in wild type legume grains

promising under-utilized legume seeds could be explored in the dietary management of certain chronic diseases such as diabetes, obesity, cancer, cardiovascular diseases, *etc*., after conducting bioavailability studies with suitable *in vivo* model. Identification of phenolic fractions of the wild legume grains is in progress with LC-MS technique.

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RESEARCH: BIO-ENGINEERING

STRAIN IMPROVEMENT OF ALKALINE PROTEASE FROM *TRICHODERMA REESEI* MTCC-3929 BY PHYSICAL AND CHEMICAL MUTAGEN

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ABSTRACT

The purpose of the present investigation is to enhance alkaline protease production by subjecting indigenous protease producing strain Trichoderma reesei MTCC-3929 to improvement by random mutagenesis by ultra-violet (UV) irradiation and N-Methyl-N'-nitro-N-nitroso guanidine (NTG) treatment. Mutants were screened as protease producers on the basis of zone of clearance on skimmed milk agar plates. UV-8 mutant showed 9 mm clear zone diameter and activities of 199.6 and 552.6 U/ml for submerged fermentation (Smf) and solid state fermentation (SSF), respectively. UV-8 further mutated by NTG to produced NTG-17 mutant with zone of clearance 13mm diameter. Compared to wild strain, NTG-17 mutant was found to produce 2.6 and 2.2-fold more activities in SmF and SSF, respectively. Thus these findings have more impact on enzyme economy for biotechnological applications of microbial proteases.

Keywords: *Trichoderma reesei; strain improvement; UV-NTG mutation; alkaline protease*

[I] INTRODUCTION

Proteases, which account for about 60% of total enzyme market and among the most valuable commercial enzymes, are the single largest class of enzymes occupying a pivotal position due their wide application in the industrial processes [1]. Plants, animals and microbial sources are employed for protease production. Microbes serve as the preferred source of proteases because of their rapid growth, the limited space required for their cultivation, and the ease with which they can be genetically manipulated to generate new enzymes with altered properties. Microbial extracellular proteases are important enzymes and are mainly used in detergents to facilitate the release of proteinaceous stains such as blood, milk, egg and meat. Today, proteases account for approximately 40% of the total enzyme sales in various industrial market sectors, such as detergent, food, pharmaceutical, leather, diagnostics, waste management and silver recovery. This dominance of proteases in the industrial market is expected to increase further by the year 2005 [2]. However, until today, the largest share of the enzyme market has been held by detergent alkaline proteases active and stable in the alkaline pH range [3]. As only few reports are available on the use of fungal proteases in detergent industry, therefore there is a growing need to exploit fungal proteases for commercial exploitation in detergent industry.

For industrial use enzyme must be produced at low cost and should be reusable and reproducible. To achieve this many techniques have been developed for protease improvements. Strain improvement is usually done by mutating the microorganism that produces the enzyme by techniques such as classical mutagenesis, which involves exposing the microbe to physical mutagens such as X-rays, γ-rays, UV rays, *etc.*, and chemical mutagens such as NTG, EMS, etc. [4].

The mutation of fungal strain for various industrial enzymes (lipase, chitinase, cellulase, glucoamylase etc) has been widely used in many investigations [5-8]. But there was no report available on mutation studies of *Trichoderma reesei* for protease productions. Also very less work was published on protease improvement from fungi using classical mutation.

In the present study, the alkaline protease production by *T. reesei* has been improved by physical and chemical mutagen

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to exploit its use in detergent industry. The effect of mutation on fungi was evaluated in Smf and SSF.

[II] MATERIALS AND METHODS

2.1. Microorganism and growth media

Trichoderma reesei MTCC-3929 was procured from Microbial Type Culture Collection (MTCC), Chandigarh (India). It was grown on Potato Dextrose agar (PDA) at 30°C, and then stored at 4°C. PDA slants, incubated for 7 days, were used for the preparation of the inoculum.

2.2. Preparation of spore suspension

Ten milliliters of 0.85% saline containing 0.1% Tween-80 was transferred to a sporulated (7 day old) PDA slant culture of *T. reesei*. The spores were dislodged using an inoculation needle, under aseptic conditions, and the suspension was used for its spore count by serial dilution and plating on PDA agar medium.

2.3. Protease screening

Protease production by *T. reesei* (wild and mutants) were tested on skimmed milk agar plates containing (g/L) 4 potato infusion, 20 dextrose, 10 skimmed milk, 20 agar (pH 5.6).

2.4. Enzyme assay

The protease activity was determined by caseinolytic assay method of [9]. The cell free supernatant (1ml) was mixed with 4ml of casein (0.625% w/v) and incubated at 40°C for 30min. The reaction was stopped by addition of 5ml of 5% trichloroacetic acid. Enzymatically hydrolyzed casein was measured by modified Folin Ciocalteu method [10], against casein treated with inactive enzyme as blank. A standard graph was generated using standard tyrosine solutions of 5–50 µg ml^{-1}. One unit of protease activity was defined as the amount of enzyme which liberated 1 µg tyrosine per min at 40°C.

2.5. Isolation and selection of mutants

2.5.1. UV irradiation

Four ml of the spore suspension containing 10^7spores/ml was pipetted aseptically into sterile petri-dish of 80 mm diameter having a flat bottom. The exposure of spore suspension to UV light was carried at distance of 30 cm away from UV lamp (15W, 2537A^0). The exposure times were 5, 10, 15, 20 and 25 min. Each UV exposed spore suspension was stored in dark overnight to avoid photo reactivation, then was serially diluted in saline and plated on PDA medium. The plates were incubated for 7 days at 30°C and the numbers of colonies in each plate were counted. Each colony was assumed to be formed from a single spore. Mutants were selected from the plates showing less than 1% survival rate and screened for protease production on skimmed milk agar plates.

2.5.2. NTG treatment

The best UV mutant (UV-8) was used for NTG treatment. The spore suspension was prepared in the same manner as described earlier and calculated the spore count. To 9 ml of spore suspension (10^6 spores/ml), 1 ml NTG solution (10 mg/ml in sterile water) was added. The reaction was allowed to proceed at 30^{0}C, 120 rpm. Samples were withdrawn from the reaction mixture at intervals of 20, 40, 60, 80 and 100 min and immediately centrifuged for 10 min at 5000 rpm and the supernatant solution was decanted. Cells were washed three times with sterile water and resuspended in 10 ml of sterile saline. The samples were serially diluted in the same saline and plated over PDA as mentioned earlier. NTG mutants were selected from the plates showing less than 1% survival rate and screened for protease production on skimmed milk agar plates.

2.6. Protease production

Hyper producing mutant of UV, NTG treatment and a wild strains were inoculated separately in Smf and SSF medium. The liquid medium was prepared which containing (g/l) 5 wheat bran, 10 soybean meal, 0.1 yeast extract, 2 KH_2PO_4, 4 K_2HPO_4, 0.5 NaCl, 0.1 $MgSO_4$, 2 $CaCl_2$ (pH 7) and the solid medium contained 10 g wheat bran with 10 ml water. Both fermentations were performed at 30^{0}C up to 5 days under shaking and static conditions for Smf and SSF, respectively. For solid medium, the fermented koji was mixed with 100 ml water and kept for 2h at room temperature. After 2h, it was filter off through cotton filter. The filtrate was used for protease activity measurements.

2.7. Statistical analysis

All values presented here are the average values of triplicate analysis ± standard deviation (SD).

[III] RESULTS AND DISCUSSION

3.1. UV Mutation

The UV treatment with fungal spores showed 1% survival when treated for 20 min **[Figure-1]**. The plates having less than 1% survival rate was observed at 20 min UV exposure time. Hopwood et al., suggested that 99.9% kill is best suited for strain improvement as the fewer survivors in the treated sample will have undergone repeated or multiple mutations which may lead to the enhancement in the productivity of the culture [11].

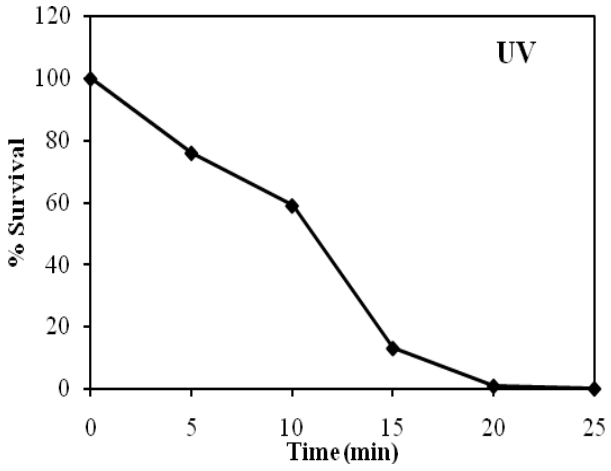

Fig: 1. UV irradiation on survival of *T. reesei* at different time (30 cm distance, 4 ml spore suspension)

From UV mutagenesis total 20 mutants were selected on the basis of different morphology, shapes and colored mutants.

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Out of 20 mutants, only two mutants (UV-3 and UV-8) showed higher clear zone diameter than wild type **[Table-1]**. UV-8 showed maximum protease activities of 199.6±6.5U/ml (in Smf) and 552.6±3.5U/ml (in SSF), respectively. The wild strain produced protease activities of 121.3±4.1U/ml (in Smf) and 344.3±5.6U/ml (in SSF), respectively. Overall, 1.6-fold protease activity was enhanced from UV-8 mutant in Smf as well as SSF, when compared with wild strain. Djamel et al. reported hyper-producing acid protease UV mutant (S08M4) that produced 1400U/ml in liquid fermentation [12]. UV mutation was also used for production of alkaline protease from *Bacillus* sp. [13].

Table 1. UV mutant and this protease activity

Isolates	Zone of clearance (MM)	Protease activity (U/ml)	
		[b]SmF	[c]SSF
Wild strain	5	121.3±4.1[e]	344.3±5.6
[a]UV-1	4	102.3±5.8	245.6±4.5
UV-2	3	77±5.1	154.3±5
UV-3	6	126.3±8.7	349±2.6
UV-4	1	35.6±6.1	84.6±3.7
UV-5	5	111±4.3	203.6±2.5
UV-6	5	83.69.2	187.3±4
UV-7	[d]+	10±2.6	41.6±4
UV-8	9	199.6±6.5	552.6±3.5
UV-9	+	0	8.3±2
UV-10	+	0	20±3.6
UV-11	4	84±5.5	237.6±3.5
UV-12	3	71.6±6.6	179±6.5
UV-13	3	66.6±5.5	195.3±4.9
UV-14	1	19±4.5	81.6±4
UV-15	2	41.6±3.5	142±3.6
UV-16	5	98±6	302.6±3.7
UV-17	1	7.6±1.5	36.6±2.3
UV-18	2	20.6±2.3	70.6±2.5
UV-19	3	40.3±2.5	159.3±3.5
UV-20	+	0	14.3±2.5

[a]UV-Ultra-violet, [b]Smf-submerged fermentation, [c]SSF-solid state fermentation, [d]protease positive, [e]Mean ± SD

3.2. NTG mutation

The UV mutant (UV-8) was selected and was subjected for further strain improvement by NTG treatment. NTG was considered to be very effective chemical mutagen. The 1% survival was observed in between 60-80 min incubation **[Figure-2]**.

Total 20 mutants were selected on SMA plates. Among 20 mutants, one mutant (NTG-17) showed maximum clear zone (13 mm diameter) than UV-8 mutant while 8 mutants showed higher clear zone diameter than wild strain. NTG-17 showed maximum protease activities of 318.3±2.5U/ml (in Smf) and 747.3±3.2U/ml (in SSF), respectively. The protease yield of NTG-17 mutant was 1.6 and 1.4-fold higher in Smf and SSF than UV-8 mutant **[Table-2]**. When compared to wild strain, NTG mutant showed 2.6 and 2.2-fold higher protease activities in Smf and SSF. Likewise, but higher fold (3.5) protease activities were reported by UV/NTG mutant of *Bacillus pumilus* [14]. Ryden et al. reported an increase in protease production of 3-10 folds by using a *Staphylococcus aureus* mutant generated by NTG [15].

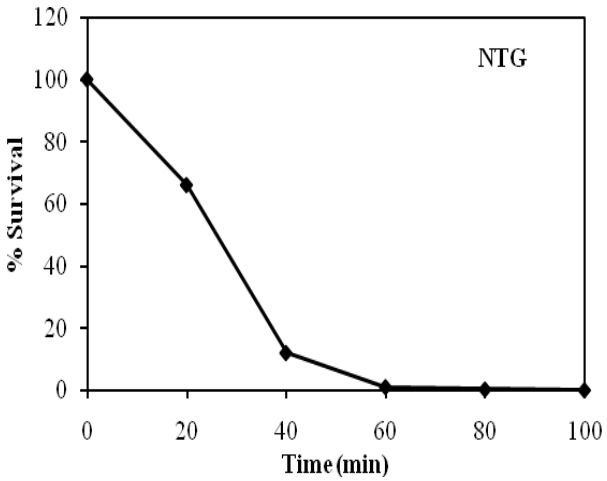

Fig: 2. NTG treatment on survival of UV mutant of *T. reesei* (UV-8) at different time (NTG, 3 mg/ml, 10 ml spore suspension)

Table 2. NTG mutant and this protease activity

Isolates	Zone of clearance MM)	Protease activity (U/ml)	
		[c]SmF	[d]SSF
Wild strain	5	124.6±4.1[e]	348.6±7
[a]UV-8	9	196.3±8.5	549.3±3.5
[b]NTG-1	4	116.3±2.5	324.3±7.5
NTG-2	5	125.3±4	355.6±0.5
NTG-3	6	145.3±3.2	402.3±3.2
NTG-4	5	124.3±4.7	350.3±1.5
NTG-5	9	190.6±2.8	482.6±5
NTG-6	3	83.6±4.5	201.6±2
NTG-7	1	37.6±4.1	97.3±3.7
NTG-8	2	46±2	145.3±3.2
NTG-9	6	151.6±3	246.6±3
NTG-10	4	112±0.5	135±3
NTG-11	3	84.3±3.5	83.3±5.1
NTG-12	4	121.6±1.5	145.6±1.5
NTG-13	2	36±3	121±2
NTG-14	6	143.6±2.5	254±5
NTG-15	4	43±2	341±3
NTG-16	7	180.6±1.5	336.3±3
NTG-17	13	318.3±2.5	747.3±3.2
NTG-18	2	42.6±1.5	144.3±1.5
NTG-19	5	121.3±3.2	336.3±2.5
NTG-20	6	146.6±4.9	384.3±4.5

[a]UV-Ultra-violet, [b]NTG- N-Methyl-N'-nitro-N-nitroso guanidine, [c]Smf-Submerged fermentation, [d]SSF-solid state fermentation, [e]Mean ± SD

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To the best of our knowledge there was no report available on strain improvement of *T. reesei* for protease production. Improvement of microbial strains for the overproduction of industrial products has been the hallmark of all commercial fermentation processes. Such improved strains can reduce the cost of the processes with increased productivity and may also possess some specialized desirable characteristics. Effectiveness of UV Irradiation (physical mutagen) and NTG treatments (Chemical mutagen) in strain improvement for enhanced protease productivity was demonstrated in the present investigation. It is hoped that the high yielding fungal mutant strain of the isolate *T. reesei* (NTG-17) can be exploited commercially for large-scale industrial production of protease.

[IV] CONCLUSIONS

A mutant, NTG-17 was obtained from a Mutant UV-8 of *T. reesei* ATCC-3929. It was screened as best potential because of its enhanced zone of clearance on skimmed milk agar plate. It was developed using UV irradiation followed by NTG treatment of the spores Compared to wild strain, NTG-17 mutant showed 2.6 and 2.2-fold higher protease production in Smf and SSF, respectively. Thus the selected mutant has potential in minimizing the cost of enzyme for its biotechnological applications.

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REVIEW: PHARMACOLOGY

EFFECT OF ASPIRIN ON HEMOSTASIS: SYNERGISM OR ANTAGONISM WITH NON STEROIDAL ANTI-INFLAMMATORY AGENTS

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ABSTRACT

Aspirin and the other non steroidal anti-inflammatory drugs (NSAIDs) are among the most often prescribed medicines. Co-administration of aspirin and NSAIDs is also common especially among patients suffering from arthritis and cardiovascular disease. An interaction between aspirin and NSAIDs could affect both the antiplatelet effect of aspirin and the safety of these agents in case of co-administration. For example, co-administration of aspirin with another NSAID could produce a pharmacodynamic interaction with subsequent enhancement or inhibition of antiplatelet effect of aspirin. On the other hand, such a pharmacodynamic interaction could result in increased incidence of gastrointestinal and non gastrointestinal haemorrhage. This review aimed to summarize available data on the possible pharmacodynamic interaction between aspirin and NSAIDs and the clinical consequences of such an interaction. Databases were searched electronically for relevant trials. Identified data were quite limited. Theoretically at the molecular level, a competitive interaction between aspirin and NSAIDs could be anticipated in case of prior administration of a NSAID. However, in vitro data indicate that concurrent administration of aspirin and diclofenac potentiates the inhibition of platelet aggregation. Yet, in vivo studies have failed to prove competitive interaction between aspirin and diclofenac in platelet aggregation, or suggest minimal effect of diclofenac on platelet aggregation, when administered concurrently with aspirin. Retrospective studies based on prescription databases have suggested that ibuprofen counteracts the antiplatelet effect of aspirin. There is limited evidence on how NSAIDs affect platelet aggregation in vivo when they are given together with aspirin according to a regular clinical schedule e.g. a morning dose of aspirin and repeated doses of NSAIDs during the day. Existing data suggest an interaction between aspirin and NSAIDs on haemostasis. However, there is controversy regarding the direction of this interaction, i..e. synergism or antagonism. Since, co-administration of aspirin and NSAIDs is quite common in people with comorbidities, further research is needed to clarify the clinical significance of this interaction.

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Keywords: *aspirin; NSAIDs; pharmacodynamic interaction; antiplatelet action; adverse drug events*

[I] INTRODUCTION

Aspirin and the other non steroidal anti-inflammatory drugs (NSAIDs) are among the most often prescribed medicines. Co-administration of aspirin and NSAIDs is also common especially among patients suffering from arthritis and cardiovascular disease. Daily doses of 75-125 mg of aspirin are recommended for individuals at high risk for cardiovascular disease. When drugs with similar pharmacologic effects are administered concurrently, an additive or synergistic interaction is usually seen. An antagonistic interaction is also possible, but not common. On the other hand the combined use of two or more drugs, each of which has toxic effects on the same organ can greatly increase the possibility of such an organ damage. An interaction between aspirin and NSAIDs could affect both the antiplatelet effect of aspirin and the safety of these agents in case of co-administration.

For example, co-administration of aspirin with another NSAID could produce a pharmacodynamic interaction with subsequent enhancement or inhibition of antiplatelet effect of

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aspirin [1]. On the other hand, such a pharmacodynamic interaction could result in increased incidence of gastrointestinal and non gastrointestinal haemorrhage [1]. The possibility, mechanisms and clinical significance of such an interaction have not been fully investigated. This study aimed to review the possible pharmacodynamic interaction between aspirin and NSAIDs and the clinical consequences of such an interaction.

[II] CO-ADMINISTRATION OF NSAIDS AND LOW DOSE ASPIRIN

Data were quite limited. A few randomized controlled trials were identified and all of them included a small number of healthy volunteers. Catella-Lawson *et al.*[2] performed a randomized cross over study with single doses of aspirin and ibuprofen and a parallel group study with multiple doses of aspirin and ibuprofen. Serum thromboxane B2 levels (an index of cyclo-oxygenase I activity in platelets) and platelet aggregation were maximally inhibited 24 hours after aspirin in the patients who took aspirin before any dose of any single drug, as well as in those who took rofecoxib or acetaminophen before aspirin. In contrast, in subjects who took 400 mg of ibuprofen two hours before taking 81 mg of aspirin, thromboxane B2 formation was inhibited by only 53% on day 7. The authors concluded that co-administration of NSAIDs with aspirin may interfere with the irreversible antiplatelet function of aspirin resulting thus in attenuation of the antiplatelet effect [2].

Another RCT, that included eleven healthy volunteers, investigated the influence of co-administration of aspirin with diclophenac, naproxen and acetaminophen on platelet aggregation. They demonstrated, that co-administration of aspirin with NSAIDs may interfere with platelet aggregation at the beginning of the treatment, with naproxen having an additional anti-aggregatory effect to that brought by a single dose of 100 mg aspirin and with diclofenac decreasing the antiagregatory effect of aspirin. The effect was lost after 4 days and the authors concluded that a regular daily administration of NSAID does not have an effect on platelet aggregation [3]. Capone *et al.*[4] investigated the pharmacodynamic interaction of naproxen with low dose aspirin in four healthy volunteers, who received aspirin (100mg per day) for six days and then the combination of aspirin and naproxen for further six days, aspirin two hours before naproxen (500 mg twice daily) . Following a washout period of 14 days, naproxen was given before aspirin for further six days. Ex vivo markers of platelet function were measured. Co-administration of naproxen did not significantly alter the antiplatelet action of aspirin.

On the contrary, in the study of Gladding *et al.*[5], ibuprofen, indomethacin, naproxen and tiaprofenic acid blocked the antiplatelet effect of aspirin. Gengo *et al.*[6] investigated the interaction of ibuprofen and low dose aspirin in healthy volunteers and the interaction of ibuprofen or naproxen with aspirin in a confirmatory study of 28 patients taking low dose aspirin for stroke prevention. The data of both studies suggested that ibuprofen prevented the irreversible inhibition of platelet aggregation produced by low dose aspirin. However, it seems that the type of interaction between aspirin and NSAIDs may differ among different NSAIDs. Thus, in vivo studies have failed to prove competitive interaction between aspirin and diclofenac in platelet aggregation, or suggest minimal effect of diclofenac on platelet aggregation, when administered concurrently with aspirin [7,8]. Observational studies have conflicting results. Retrospective studies suggest that ibuprofen counteracts the antiplatelet effect of aspirin [9,10]. However, the strength of evidence is limited, since data came from prescription databases.

[III] MECHANISM OF PHARMACODYNAMIC INTERACTION

The most predicted hematological adverse reaction of aspirin and NSAIDs is due to inhibition of cyclo-oxygenase I. COX-I catalyzes the transformation of membrane bound arachidonic acid to thromboxane A2, a platelet agonist with resultant reduced platelet adhesiveness and prolongation of bleeding time. According to crystallographic data, aspirin inhibits cyclo-oxygenase by irreversible acetylation of a serine residue in vicinity with the catalytic site of the enzyme, while NSAIDs bind to the same hydrophobic channel of COX I in the vicinity of aspirin [11,12].

Because the ability of aspirin to acetylate a critical serine residue at the apex of the COX channel is dependent on its initial binding to arginine-120, a common docking site for all NSAIDs, the stronger binding affinity of nonaspirin NSAIDs might preclude aspirin from permanently modifying platelet COX-1. Thus, theoretically in molecular level, a competitive interaction between aspirin and NSAIDs could be anticipated in case of prior administration of a NSAID. On the other hand, highly selective COX-2 inhibitors (coxibs) are less likely to interfere with the antiplatelet effect of aspirin than conventional NSAIDs because of their limited interaction with platelet COX-1.

However, in cellular and organism level, synergistic or even additive effect between aspirin that irreversibly blocks COXI activity and diclofenac that reversibly blocks COXI activity could be anticipated. First of all, low doses of aspirin are associated with high inhibition of COXI as demonstrated by almost total inhibition of serum thromboxane [13]. However, there is residual platelet reactivity in patients treated with aspirin, the antiplatelet action of aspirin seems to be dose-related, implying thus, that aspirin as an antiplatelet might affect other targets besides COXI [14]. For example, reactive oxygen species seem to play a significant role in the regulation

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of platelet activation [15]. Aspirin has been shown to modulate reactive oxygen species through its pro-oxidant or antioxidant actions [16]. Additionally, aspirin has been shown to enhance fibrinolysis and suppress plasma coagulation, although the clinical significance of these is not determined. There is limited evidence on how NSAIDs affect platelet aggregation in vivo when they are given together with aspirin according to a regular clinical schedule e.g. a morning dose of aspirin and repeated doses of NSAIDs during the day.

[IV] NSAIDS AND ANTIPLATELET ACTION

The antiplatelet action of NSAIDs is a matter of controversy. It has been demonstrated that NSAIDs have in vitro antiplatelet action. However, in *ex vivo* studies, there is extensive variability on the extent and duration of the effects of NSAIDs on platelet aggregation and on bleeding times. The antiplatelet effects of long acting NSAIDs like piroxicam persist for several days after the drug is stopped. Platelet aggregation is inhibited within 2 hours after a single dose of ibuprofen, but the effect is lost within 12 hours. High dosages of ibuprofen cause slight but significant prolongations of bleeding time for several hours while lower doses (e.g. 200 mg three times daily) may not affect the bleeding time. In general, most NSAIDs cause transient dose dependent prolongations of bleeding time, without exceeding the upper limit of normal range for bleeding time [17].

The clinical significance of the antiplatelet action of NSAIDs is not determined [18-20]. Clinical trials have suggested cardioprotective effect of naproxen, flurbiprofen and diclofenac due to antiplatelet action [21-24]. However, other papers imply that NSAIDs do not have an antiplatelet action, since: first, the relation between the inhibition of platelet cyclo-oxygenase I dependent thromboxane A2 generation and the inhibition of thromboxane dependent platelet function is non linear and second, NSAIDs inhibit platelet cyclo-oxygenase I, but the inhibition lasts for only a part of the dosing interval. It has been proposed that platelet COX-I has to be almost completely (>95%) and continuously inhibited ex vivo throughout the dosing intervals to translate to a detectable cardiovascular protection. However, an antiplatelet action has been proposed for naproxen. It has been suggested that naproxen inhibits platelet cyclo-oxygenase I for the whole duration of the dosing interval. Ex vivo studies have shown that naproxen at therapeutic doses of 500 mg twice daily gets into the functionally relevant range of inhibition of platelet COX I activity (>95%), at the end of the dosing interval in some subjects [25]. Additionally, NSAIDs might affect platelet function in a cyclo-oxygenase independent mechanism. A recent paper suggests antagonism of thromboxane receptors as a novel mechanism of action of diclofenac [26]. Therefore, it could be proposed that COXI independent mechanisms might contribute to the pharmacodynamic interaction between aspirin and diclofenac on hemostasis.

[V] CLINICAL SIGNIFICANCE OF NSAIDs-ASPIRIN INTERACTION

The possible interaction of aspirin and NSAIDs on antiplatelet action is clinically significant not only in cardioprotection but also in aspirin and NSAIDs induced haemorrhage. If co-administration of aspirin and NSAIDs enhances antiplatelet action of aspirin alone, increased frequency of gastrenteric and non gastrenteric hemorrhage is expected. Indeed, intracerebral hemorrhage is the most serious spontaneous hemorrhage caused by aspirin. It seems that patients prescribed non aspirin NSAIDs are not at an overall increased risk of being hospitalized for intracerebral hemorrhage. However, existing data are quite scarce, and there is no estimation about the risk in case of co-administration of aspirin and NSAIDs [27].

[VI] CONCLUSION

Existing data suggest an interaction between aspirin and NSAIDs on haemostasis. However, there is controversy regarding the direction of this interaction, i..e. synergism or antagonism. Since, co-administration of aspirin and NSAIDs is quite common in people with comorbidities, further research is needed to clarify the clinical significance of this interaction.

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