J.Pharm.Chem CODEN: JPCOCM

Journal of Pharmacy and Chemistry

(An International Research Journal of Pharmaceutical and Chemical Sciences)
Indexed in Chemical Abstract and Index Copernicus

www.stfindia.com
www.jpc.stfindia.com

J.Pharm.Chem CODEN: JPCOCM

Journal of Pharmacy and Chemistry

(An International Research Journal of Pharmaceutical and Chemical Sciences)

Volume 7 • Issue 4 • October – December 2013

Contents

VIEWS

ISSN 0973 – 9874

Reactive Oxygen Species and Antioxidant Defense in Response to Abiotic Stresses in Plants

K.V. MADHUSUDHAN*[1] AND C. SUDHAKAR[2]

[1] Government Degree College for Men, Kurnool, Andhra Pradesh, India
[2] Department of Botany, S.K.University, Anantapur, Andhra Pradesh, India

ABSTRACT

Abiotic stress is a serious threat to agriculture affecting plant growth and productivity, mediated at least in part, by an enhanced generation of reactive oxygen species (ROS). Several ROS are also continuously produced as normal by-products of aerobic metabolism and the four major active oxygen species includes superoxide anion (Radicals) ($O_2^{\bullet-}$), singlet oxygen ($^1O_2$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($OH^{\bullet}$). These are highly reactive in the absence of any protective mechanism and can seriously disrupt normal metabolism through oxidative damage to membrane lipids, proteins, carbohydrates, pigments and nucleic acids, which ultimately results in the cell death. On the other hand, ROS also act as signaling molecule to control and regulate various biological processes including pathogen defense, programmed cell death, cell cycle, stress responses, growth and development. The major potential sources of ROS in plants are chloroplasts, mitochondria and peroxisomes. Plants have mechanisms to deal with them in normal conditions, controlling the formation and removal rates. However, under stress conditions, the balance between production and elimination of ROS is disturbed and this results in oxidative stress. Plants have developed several antioxidation strategies by an array of enzymatic and non-enzymatic antioxidants, that can protect cells from oxidative damage and scavenge toxic ROS that are produced in excess of those normally required for various metabolic reactions. An overview of the literature is presented in terms of generation of ROS and it's role as both deleterious and beneûcial species. The antioxidative mechanism in relation to the oxidative load in the context of stress tolerance is also discussed.

Key words : Reactive oxygen species, Abiotic stress, Antioxidant defense, Oxidative stress.

Abbreviations: AA, ascorbic acid; ABA, abscisic acid; Apo, apoplast; APX, ascorbate peroxidase; CAT, catalase; Chl, chloroplast; CW, cell wall; DHA, dehydroascorbate; DHAR, dehydroascorbate reductase; DNA, deoxyribonucleic acid; ET, electron transport; FA, Fatty acid; GSH, reduced glutathione; GSSG, oxidized glutathione; GPOX, guaiacol peroxidase; GPX, glutathione peroxidase; GR, glutathione reductase; GST, glutathione-s-transferase; HO, heme oxygenase; H_2O_2, hydrogen peroxide; $OH^{\bullet}$,hydroxyl radical; MDA, malondialdehyde; MDHA, monodehydroascorbate; MDHAR, monodehydroascorbate reductase; Mit, mitochondria; NAD, nicotinamide adenine dinucleotide; NADP, nicotinamide adenine dinucleotide phosphate; NO, nitric oxide; PCD, programmed cell death; Per, peroxisome; $OONO^H$, peroxynitrate; $HO_2^{\bullet}$, perhydroxyl radical; PM, plasma membrane; POX, peroxidase; PUFAs, polyunsaturated fatty acids; ROS, reactive oxygen species; $O_2^{\bullet-}$, superoxide radical; $^1O_2$, singlet oxygen; SA, salicyclic acid.

Introduction

Abiotic stresses, such as drought, salinity, extreme temperatures, chemical toxicity and oxidative stress are the primary causes of crop loss worldwide, reducing average yields for most major crop plants by more than 50% [1]. As an unfortunate consequence of aerobic life, reactive oxygen species are formed by partial reduction of molecular oxygen. ROS are inevitable byproducts of various metabolic processes that are localized in different cellular compartments such as chloroplast, mitochondria and peroxisomes [2], and plants have mechanisms to deal with them in normal conditions, controlling the formation and removal rates. Under stress conditions, ROS production can exceed the plant's defense mechanism, an imbalance in intracellular ROS content is established and this results in

*Address for correspondence: botanymadhu@gmail.com

an oxidative stress [3]. ROS include $O_2^{\bullet-}$, $^1O_2$, H_2O_2, $OH^{\bullet}$ which can pose a threat to cells by causing peroxidation of lipids, oxidation of proteins, damage to nucleic acids which ultimately leads to cell death [4]. On one hand, plants need to control the levels of these oxidants because of their harmful nature, but on the other hand, they also use ROS as signaling molecules especially in response to various stresses or threats to the plant integrity, as pathogen attacks, or non-optimal growth conditions. Because of the multifunctional roles of ROS, it is necessary for the cells to control the level of ROS tightly to avoid any oxidative injury and not to eliminate them completely. Plants possess very efficient enzymatic and non-enzymatic antioxidant defense systems that can protect cells from the oxidative damage. The enzymatic antioxidants include superoxide dismutase (SOD), catalase (CAT), Guaiacol peroxidase (GPOX), enzymes of ascorbate-glutathione (AA-GSH) cycle such as ascorbate peroxidase (APX), monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR), and glutathione reductase (GR) [4]. Ascorbate (AA), glutathione (GSH), carotenoids, tocopherols and phenolics serve as potent non-enzymatic antioxidants within the cell [2]. It has also been reported that plants with high levels of antioxidants, whether constitutive or induced by various environmental stresses, have a greater resistance to oxidative damage [5,6]. This article reviews briefly on types of ROS, their effects and production sites, with a special focus on the role of the active oxygen scavenging systems in combating oxidative stress.

ROS Biochemical properties

Atomic oxygen is a free radical and molecular oxygen is a (free) bi-radical. This feature makes oxygen paramagnetic; it also makes oxygen very unlikely to participate in reactions with organic molecules unless it is activated. ROS are produced from the excitation of O_2 to form $^1O_2$ or from the transfer of one, two or three electrons to form $O_2^{\bullet-}$, H_2O_2 and $OH^{\bullet}$, respectively [7]. ROS are capable of unrestricted oxidation of various cellular components and can lead to the oxidative destruction of the cell. It has been estimated that about 1% of oxygen consumed by plants is diverted to produce ROS in various sub cellular loci [8]. $^1O_2$, is a highly destructive species, reacting with most biological molecules at near diffusion-controlled rates [9]. It can last for nearly 4¼s in water and 100¼s in a non polar environment [10].$^1O_2$ targets the conjugated double bonds present on polyunsaturated fatty acids (PUFAs) leaving a specific footprint in the cell [11] that can be followed by the detection of several aldehydes like malondialdehyde (MDA) formed by PUFA peroxidation. A single electron reduction of O_2 results in the generation of $O_2^{\bullet-}$ and the majority of O_2 reduction *in vivo* is thought to proceed via reduced ferredoxin, which reduces molecular oxygen to the superoxide radical (Mehler reaction in chloroplast). Mitochondrial electron transport chain generates $O_2^{\bullet-}$ at complex I and III. $O_2^{\bullet-}$ can also be produced by NADPH oxidase in the plasma membrane and xanthine oxidase in peroxisomes [11]. $O_2^{\bullet-}$ is a moderately reactive, short-lived with a half-life of 2-4 µs; therefore, it cannot cross phospholipid membranes and can be easily dismuted to H_2O_2 [12]. Alternatively, $O_2^{\bullet-}$ reduces quinones and transition metals complexes of Fe^{3+} and Cu^{2+}, thus affecting the activity of metal-containing enzymes. $O_2^{\bullet-}$ can be protonated to form perhydroxyl radical ($HO_2^{\bullet}$), which can cross bio membranes and subtract hydrogen atoms from PUFAs and lipid hydroperoxidases, thus initiating lipid peroxidation [13]. $O_2^{\bullet-}$ can also react with another very influential signaling free radical species, $NO^{\bullet}$ to give up peroxynitrate ($OONO^{H}$). H_2O_2 is produced by the dismutation of $O_2^{\bullet-}$ in a reaction mostly catalyzed by SOD. H_2O_2 is generated in the cells under normal as well as wide range of stressful conditions. H_2O_2 is moderately reactive and is relatively long-lived molecule with a half-life of 1ms [14], and can easily cross membranes diffusing across the cell. H_2O_2 may inactivate enzymes by oxidizing their thiol groups. It has received particular attention as a signal molecule involved in the regulation of speciûc biological processes and triggering tolerance against various environmental stresses [11]. H_2O_2 can also lead to the production of $OH^{\bullet-}$, the most reactive oxidant in ROS family, via the so called Haber-Weiss/Fenton reactions, which use suitable transition metals, especially iron (Fe), at neutral pH and ambient temperature and is considered as one of the initiation radicals for lipid peroxidation [13]. $OH^{\bullet}$ is not considered to have signaling function, although its products of its reaction can elicit signaling responses. Unlike the previous ROS mentioned, there are no known enzymatic systems that are able to degrade $OH^{\bullet}$; therefore its production in excess can eventually lead to cell death [12].

ROS oxidative damage in plant cells

Mechanisms by which oxygen radicals damage membrane lipids are well accepted and consequently oxidative damage is often exclusively associated with peroxidation reactions in membrane lipids. Malondialdehyde is one of the ûnal products of peroxidation of unsaturated fatty acids in phospholipids and is responsible for cell membrane damage [13]. Two common sites of ROS attack on the phospholipid molecules are the unsaturated (double) bond between two carbon atoms and the ester linkage between glycerol and the fatty acid. The PUFAs present in membrane phospholipids are particularly sensitive to attack by ROS. An oxygen can then easily bind to that location forming a lipid peroxyl radical, that can continue and propagate the same kind of reactions [15]. Proteins can also suffer from oxidation by ROS, causing certain enzymes to lose its catalytic function. Oxidative attack on proteins results in site-specific amino acid modifications, fragmentation of the peptide chain, altered electrical charge and increased susceptibility to proteolysis. The amino acids in a peptide differ in their susceptibility to attack, and the various forms of activated oxygen differ in their potential reactivity. One of the more susceptible targets in proteins are thiol groups the oxidation of which can lead to protein

Table 1

Major ROS and their properties, production and scavenging.

ROS	Relative Reactivity	half-life & mobility	Production	Location	Scavenging
Singlet oxygen ($^1O_2$)	High	1μs, 30nm	Excited chlorophyll	Chl	Caretenoids α-tocopherol
Superoxide radical ($O_2^{\bullet-}$)	Medium	2-4μs, 30nm	Photosynthetic ET Respiration ET Xanthine oxidase NADPH oxidase	Chl Mit Per PM	Superoxide dismutases (SODs)
Hydroxyl radical ($OH^{\bullet}$)	Very high	1ns, 1nm	Haber-Weiss/ Fenton reaction	Chl, Mit, Per	Flavonoids
Hydrogen peroxide (H_2O_2)	Low	1ms, 1μm	Photosynthetic ET Respiration ETC Glycolate oxidase FA β-oxidation CW peroxidase Oxalate oxidase Amine oxidase	Chl Mit Per Per CW Apo Apo	Catalase, Peroxidases and its family of enzymes, Peroxiredozins Ascorbic acid, Glutathione

denaturation and loss of functional conformation [16]. Activated oxygen can abstract a hydrogen atom from cysteine residues to form a thiyl radical that will transform to a second thiyl radical to form disulphide bridges. Alternatively, oxygen can add to a methionine residue to form methionine sulphoxide derivatives. Also, protein oxidation leads to the production of carbonyl groups and to increased rate of proteolysis as the damaged proteins are targeted by proteolytic enzymes. Oxidative attack on DNA results in deoxyribose oxidation, strand breakage, removal of nucleotides, variety of modiûcations in the organic bases of the nucleotides, and DNA-protein cross links. Further, changes in the nucleotides of one strand can result in the mismatches with the nucleotides in the other strand, yielding subsequent mutations and genetic defects [17].

ROS as signalling molecules in plant cells

ROS act as a signaling molecules, secondary messenger, mediating the acquisition of tolerance to both biotic and abiotic stresses [7,10,15]. Plants can sense, transduce and translate ROS signal into appropriate cellular responses with the help of some redox-sensitive proteins, calcium mobilization, protein phosphorylation, and gene expression [4]. ROS also modulate the activities of many components in signaling, such as protein phosphatases, protein kinases, and transcription factors and communicate with other signal molecules and the pathway forming part of the signaling network that controls response downstream of ROS [13]. ABA induced H_2O_2 is an essential signal in mediating stomatal closure to reduce water loss through the activation of calcium-permeable channels in the plasma membrane [18]. Gravity induced auxin stimulates H_2O_2 generation to mediate gravitropism [19]. ROS appear to be involved in dormancy alleviation through ABA signaling in barley grains [20]. In incompatible plant–pathogen interactions H_2O_2 has been implicated in the elicitation of a variety of defence responses. Increase in H_2O_2 production (as a result of oxidative burst) has been noticed by action of plasma-membrane associated NADPH-dependent superoxide synthase together with apoplastic SOD during hypersensitive response [21]. H_2O_2 produced during this response (up to 15 μM) is thought to diffuse into cells and, together with salicylic acid (SA) and nitric oxide (NO), to activate many of the plant defenses, including PCD [22]. The involvement of ROS in the regulation of stomatal closure [18] and in other cellular responses involving auxin [20] might suggest that more signaling pathways involving ROS as inducers of systemic signals await discovery.

ROS scavenging antioxidative system in plant cells

Plants have developed several antioxidation strategies by an array of enzymatic and non-enzymatic antioxidants, that can protect cells from oxidative damage and scavenge toxic ROS that are produced in excess of those normally required for various metabolic reactions.

Enzymatic antioxidants

SOD, the family of metallo enzymes are the first line of defense against injury caused by ROS, catalyzing the dismutation of O_2–• to H_2O_2 and O_2 [23]. SOD removes superoxide, hence decreases the risk of hydroxyl radical formation from superoxide via the metal-catalyzed Haber-Weiss-type reaction. SOD has a metal cofactor and depending on the metal can be classified into three different groups (isoforms), localized in different cell compartments: FeSOD (chloroplasts, cytosol and mitochondria), MnSOD (mitochondria and peroxisomes), Cu/ZnSOD (chloroplast, peroxisomes, cytosol and apoplast) [4, 11]. SOD activity has been reported to increase under abiotic stresses, and exhibited tolerance towards oxidative damage [23, 24]. Catalase is predominantly found in leaf peroxisomes where it functions chiefly on H_2O_2, generated during photorespiration and/or β-oxidation of fatty acids in glyoxysomes, converts to oxygen and water [6]. CAT catalyses the hydrogen peroxide breakdown to water. CAT comprises a family of isoenzymes CAT-1, CAT-2 and CAT-3 that differed in their molecular, biochemical properties, cell or organ specific and temporal patterns of expression and also known to be differentially affected by environmental factors [24]. Peroxidases comprise a family of enzymes with different characteristics which play a crucial role in the detoxification of cellular H_2O_2, the toxic product of superoxide dis-mutation. It scavenges excess amount of hydrogen peroxide formed in plant cells under normal and stress conditions. Induction in peroxidase has been documented under various oxidative stress factors [25], and it has been linked with protection from oxidative damage, lignification and cross linking of cell wall to prevent from such adverse conditions. High POX activity and isoforms under stress conditions have been correlated with reduction in plant growth, and this reduction has been attributed to POX catalysis of feruloylation of hemicelluloses and insolubilization of hydroxyproline-rich glycoproteins causing cell-wall stiffening [26]. Unlike catalase, peroxidase requires a substrate (R) for catalysis and it represents different electron donors in their family of enzymes, guaiacol peroxidase (EC 1.11.1.7) uses mainly phenolic donors, ascorbate peroxidase uses ascorbic acid and glutathione peroxidase uses glutathione. Apart from scavenging of H_20_2, GPX also serves to detoxify products of lipid peroxidation formed due to activity of ROS. GPX decomposes peroxides to water (or alcohol) while simultaneously oxidizing GSH. GPX competes with CAT for H_20_2 as a substrate and is the major source of protection against low levels of oxidative stress. APX is involved in scavenging of H_20_2 in water-water and ascorbate-glutathione cycles and the APX family consists of at least five different iso-forms including thylakoid and microsomal membrane bound forms, as well as soluble stromal, cytosolic and apoplastic enzymes. The water-water cycle occurs in chloroplasts and is a fundamental mechanism to avoid photo oxidative damage [27], using SOD and APX to scavenge the superoxide radical and hydrogen peroxide in the location where they are produced avoiding the deleterious effects of their reactivity with other cellular components [24]. The ascorbate-glutathione cycle is an important group of reactions involved in ROS detoxification, as it converts hydrogen peroxide (formed as a consequence of an induced stress or via SOD action) and occurs in several cell compartments, like chloroplasts, cytosol, mitochondria, peroxisomes and apoplast. It uses APX and GPX as well as other enzymes like monodehydroascorbate reductase and dehydroascorbate reductase that have a role in the regeneration of the reduced form of ascorbate. Glutathione reductase, is a potential enzyme of ASH-GSH cycle and plays essential role in defense system against ROS by sustaining the reduced status of GSH. It is found in chloroplasts as well as in mitochondria and cytoplasm. Increased cellular glutathione and glutathione reductase have been reported in plants which are tolerant to abiotic stresses [24, 28]. Glutathione S-transferases are a large and diverse group of enzymes which catalyses the conjugation of electrophilic xenobiotic substrates or endogenous organic compounds with reduced glutathione. The enhancement of GST enzyme activity has been considered as a marker for plant response to multiple environmental stress [29].

Non-enzymatic Antioxidants

Ascorbic acid (AA) occurs in all plant tissues, usually being higher in photosynthetic cells. Besides its role as an enzyme substrate, it can directly scavenge $O_2^{\bullet\bullet}$, $^1O_2$ and $OH^{\bullet}$ and regenerate tocopherol from tocopheroxyl radical,

Table 2

Major ROS scavenging antioxidant enzymes and their location

Antioxidants	Reaction catalyzed	Enzyme code	Location
SOD	$O_2^{\bullet-} + O_2^{\bullet-} + 2H^+ \rightarrow 2H_2O_2 + O_2$	1.15.1.1	Cyt, Chl, Per, Mit
CAT	$2H_2O_2 \rightarrow O_2 + 2H_2O$	1.11.1.6	Per, Gly Mit
APX	$H_2O_2 + AA \rightarrow 2H_2O + DHA$	1.11.1.11	Cyt,Per,Chl, Mit
GPX	$H_2O_2 + 2GSH' \rightarrow 2H_2O + GSSG$	1.11.1.7	Chl, Cyt, Mit, ER
MDHAR	$MDHA + NAD(P)H \rightarrow AA + NAD(P)^+$	1.6.5.4	Chl, Mit, Cyt
DHAR	$DHA + 2GSH \rightarrow AA + GSSG$	1.8.5.1	Chl, Mit, Cyt
GR	$GSSG + NAD(P)H' \rightarrow 2GSH + NAD(P)^{+\cdot}$	1.6.4.2	Cyt, Chl, Mit

thus providing membrane protection [2,3]. AA also acts as co-factor of violaxanthin de-epoxidase, thus sustaining dissipation of excess excitation energy [30]. Glutathione is a tripeptide (α glutamylcysteineglycine) localized in most of the cellular components such as chloroplasts, mitochondria, endoplastic reticulam, vacuole and cytosol and performing multiple functions [31]. It has been suggested that the GSH/GSSG ratio, indicative of the cellular redox balance, may be involved in ROS perception [3]. It scavenges H_2O_2 and reacts non enzymatically with $O_2^{\bullet-}$, $^1O_2$ and $OH^{\bullet}$. GSH regenerates AA, via the ascorbate–glutathione cycle [32], detoxifies xenobiotics and protects plants from heavy metals by performing as phytochelators [12]. It is generally believed that maintaining a high reduced per oxidized ratio of ascorbic acid and glutathione is essential for the proper scavenging of ROS in cells. This ratio is maintained by GR, MDAR and DHAR using NADPH as reducing power [31]. Tocopherols (α, β, γ, and δ) represent a group of lipophilic antioxidants are known to protect lipids and other membrane components by physically quenching and chemically reacting with O_2 in chloroplasts, thus protecting the structure and function of Photo system II [32]. Tocopherols prevent the chain propagation step in lipid auto oxidation which makes it an eûective free radical trap [12], and has been shown to induce tolerance to various abiotic stresses [33]. Phenolics are diverse secondary metabolites (flavonoids, tannins, hydroxy-cinnamate esters, and lignin) which possess antioxidant properties. They are abundantly found in plant tissues and can chelate transition metal ions, can directly scavenge molecular species of active oxygen, and can inhibit lipid peroxidation by trapping the lipid alkoxyl radical [12]. They also modify lipid packing order and decrease fluidity of the membranes. There is some evidence of induction of phenolic metabolism in plants as a response to multiple stresses [34]. Flavonoids form a great share of antioxidant-relevant phenolic compounds and have been shown to have a protective role against several stresses both by themselves and in conjugation with peroxidases [26]. Anthocyanins, a type of flavonoids (glucosides of anthocyanidins) present in the vacuoles, have an antioxidative capacity [34], but its location in the cell prevents them to contact directly with ROS production sites, although its levels have been reported to increase under cadmium stress [35]. Carotenoids have an important protective role during photosynthesis as these molecules can quench the excited states of chlorophyll in order to avoid the production of singlet oxygen [12]. As a consequence, the carotenoid molecules become themselves excited but this is not a big problem as they don't have enough energy to form this ROS species [36]. Thiols can play an important antioxidative role, protecting membrane lipids. Lipoic acid both in its reduced and oxidized form, is reported to have antioxidative properties due to its direct scavenging of ROS. It is also able to chelate several metal ions that induces oxidative stress [37] and thus can have an important role in cell protection. Proline, an amino acid regulates cytosolic acidity and maintains NAD+/NADH ratios, enhances photosystem II-mediated photochemical activity in isolated thylakoid membranes and prevents the photoinhibitory loss of photochemical activity by producing a reduction in lipid peroxidation [38]. Accumulation of proline has been reported to improve plant resistance to oxidative stress by scavenging ROS (namely by quenching singlet oxygen and hydroxyl radicals), increasing the activity of antioxidative enzymes and protecting them and maintaining redox homeostasis [39]. Glycinebetaine plays a major role in protecting membranes and macromolecules from damaging effect of stress [40]. The concentration of some polyamines has been reported to increase under abiotic stress [41]. However it is still not clear the exact role these compounds play in heavy metal defence, but a participation in the stabilization and protection of the membrane systems has been proposed [42]. Although soluble sugars have been linked to metabolic pathways that produce ROS, they can also have an important role in ROS scavenging mechanisms. Increased glucose levels can increase the production of NADPH (via the pentose-phosphate pathway), that is an important intermediate in the ascorbate-glutathione cycle [43] as NADPH is the primary electron donor that assures an intracellular reduction status. Both glucose and sucrose levels have been shown to increase in some plant species under certain abiotic stresses but it is not obvious that this happens due to a putative defense mechanism induction [43], although these sugars also participate in signalling mechanisms. Other sugars like raffinose and fructans (which are fructose polymers) are also reported to have a protective role of membranes against several stresses, namely freezing and drought stress [44].

Conclusions and future perspectives

Oxidative stress is a central factor in abiotic and biotic stress phenomena that occurs when there is a serious imbalance in any compartment between the production of reactive oxygen species (ROS) and antioxidant defence, capable of unrestricted oxidation of cellular components and can lead to the oxidative destruction of cell. However, despite their potential for causing harmful oxidations, it is now well established that ROS are also powerful signaling molecules that are involved in the control of plant growth and development as well as priming acclimatory responses to stress stimuli. Thus, it is important for cells to control the concentration of ROS tightly, but not to eliminate them completely. Plants protect themselves by scavenging and disposing of these reactive molecules by use of an enzymatic and non-enzymatic antioxidant system present in several subcellular compartments. Although rapid progress has been made in oxidative stress in recent years, there are many uncertainties and gaps of regulatory networks that control the production and scavenging of ROS and coordination of antioxidants and related compounds with growth and development of plants in a constantly changing environment. Thus a comprehensive analysis of gene

expression coupled with metabolomics, and proteomics will provide an integrated understanding of physiological and biochemical basis of stress response in plants.

References

1. Vij S, Tyagi A K. Plant Biotechnol J 2007; 5:361.
2. Gill SS, Tuteja N. Plant Physiol Biochem 2010; 48:143.
3. Maheshwari R, Dubey RS. Plant Growth Regul 2009; 59:37.
4. Sharma P, Jha AB, Dubey RS, Pessarakli M. J Bot 2012; 217307:51.
5. Chen Q, Zhang M, Shen S. Acta Physiol Plantarum 2010; 33:273
6. Mittler R. Trends Plant Sci 2002; 7:405.
7. Asada K, Takahashi M, Photoinhibition: Topics of Photosynthesis, 9th ed. Elsevier,Amsterdam 1987:227.
8. Asada K. Plant Physiology 2006; 141:391.
9. Foyer CH, Lelandais M, Kunert KJ. Physiol Planta1994; 92: 696.
10. Moller IM, Jensen PE, Hansson A. Ann Rev Plant Bio 2007;5:459.
11. Karuppanapandian T, Moon J, Kim C, Manoharan K, Wook, K. Aust J Crop Sci 2011;5:709.
12. Halliwell B, Gutteridge JMC.Free Radicals in Biology and Medicine 4th ed. Oxford University Press, Oxford, 2006.
13. Garg N, ManchandaG. Plant Biosystems 2009; 143: 81.
14. Blokhina O, Fagerstedt KV. Physiol Planta 2010; 138: 4:447.
15. Moller IM, Jensen PE, Hansson A. Ann Rev Plant Biol 2007; 58:459.
16. Tuteja N, Singh M B, Misra MK, Bhalla PL, Tuteja R. Crit Rev Biochem Molec Biol, 2001; 36:337.
17. Xiong, Schumaker KS, Zhu JK. Plant Cell 2002; 14:165.
18. Joo JH, Bae YS, Lee JS. Plant Physiol 2001; 126:1055.
19. Bahin E, Bailly C, Sotta B, Kranner I, Corbineau F, Leymarie J. Plant Cell Environ 2011; 34:980.
20. Grant JJ, Loake GJ. Plant Physiol. 2000;124:21.
21. Mittler R. et al. Proc Natl Acad Sci USA 1999; 96:165.
22. Denness L McKenna JF, Segonzac C. Plant Physiol 2011; 156:1364.
23. Agarwal S, Shaheen R.Braz J Plant Physiol 2007; 19:149.
24. Thippeswamy, Ph.D. Thesis, SK University, Anantapur, India :2007.
25. Mika A, Minibayeva F, Beckett R, Lüthje S. Phytochem Rev 2004;3:173.
26. Sofo A, Dichio B, Xiloyannis C, Masia A. Funct Plant Biol 2005; 32: 45.
27. Apel K, Hirt H. Annu Rev Plant Biol 2004;55:373.
28. Edwards R, Dixon DP, Walbot V. Trends Plant Sci 2000 5:193.
29. Balestrasse KB, Noriega GO, Batlle A, Tomaro ML. Free Radical Res 2005; 39:145.
30. Noctor G, Foyer CH. Ann Rev Plant Physiol Plant Molecular Bio 1998; 49:249.
31. Ivanov BN, Khorobrykh S. Antioxidants and Redox Signaling 2003; 5:43.
32. K. Yamaguchi-Shinozaki K, Shinozaki K. Plant Cell 1994; 6:251.
33. Michalak A. Polish J Environ Studies 2006; 15: 523.
34. Kahkonen MP Heinonen MJ. Agri Food Chem 2003; 51:628.
35. Mobin M, Khan NA. J Plant Physio 2007; 164: 601.
36. Taiz L, Zeiger E. Plant Physiology 3rd ed. Sunderland, MA: Sinuaer Ass, Inc.
37. Navari-Izzo, F, Quartacci MF, Sgherri C. Plant Physiol Biochem 2002; 40:463.
38. Alia, Saradhi PP, Mohanty P. Biochem Biophys Res Commun 1991; 181:1238.
39. Matysik J, Alia Bhalu B, Mohanty P. Current Sci 2002; 82:525.
40. Zhang FL, Niu B, Wang, YC, Chen F, Wang SH, Xu Y, Jiang LD, Gao S, Wu J, Tang L, Jia YJ. Plant Sci 2008; 174:510.
41. Mascher R, Lippmann B, Holzinger S, Bergmann H. Plant Sci 2002; 163:961.
42. Sharma SS, Dietz K J. J Exp Bot 2006; 57:711.
43. Couée I, Sulmon C, Gouesbet G, El Amrani A. J Exp Bot 2006; 57:449.
44. Vanden EW, Valluru R. Sucrose, sucrosyl oligosaccharides, and oxidative stress: scavenging and salvaging? J Exp Bot 2009; 60: 9.

ISSN 0973 – 9874

Formulation Design and *in vitro* Evaluation Studies of Matrix Diffusion Controlled Glipizide Transdermal Patch

NIRANJAN PANDA[1*], K.C. PANDA[1], A. VENKATESHWAR REDDY[1], G.V. SUBBA REDDY[2] AND L. RAJESH PATRO[3]

[1]Anwarul Uloom College of Pharmacy, Newmallepally, Hyderabad-500001, A.P, India.
[1*]Research scholar, Department of Pharmaceutical Sciences, JNTUA, Anantapur
[2]Department of Chemistry, JNTUA College of Engineering, Pulivendula, YSR(Kadapa) (dist), A.P.
[3]Avanthi institute of pharmaceutical sciences, Hyderabad

ABSTRACT

The aim of the present study was to design, optimize and evaluate matrix diffusion controlled release Glipizide transdermal drug delivery system using hydrophilic synthetic polymers like hydroxyl propyl methyl cellulose (HPMC K15M), poly vinyl pyrrolidone (PVP K30), poly vinyl alcohol (PVA) and hydrophobic polymer like ethyl cellulose (EC). Dimethyl sulfoxide (DMSO) was used as permeation enhancer and Polyethyline glycol (PEG 4000), as a plasticizer. Titanium dioxide was used as an opacifying agent and acetone was used as solvent. Transdermal patches were prepared by solvent casting techniques with different concentration of HPMC, EC, PVP and PVA to optimize the release rate as well as for prolonged release of drug for extended period of time. Prepared patches of all the formulations were evaluated for physicochemical parameters like weight variation, thickness, folding endurance, drug content, % moisture absorption, % elongation break test. The in-vitro diffusion study of prepared patches from all the batches were carried out by using specially fabricated Franz diffusion cell. To ensure drug excipients compatibility study both FTIR and DSC were carried out. The evaluation results of all parameters were satisfactory according to the pharmacopeia specifications. From various kinetic study it was concluded that the in vitro release follows zero order kinetic.

Key words: Transdermal patches, Controlled release dosage form, Glipizide, HPMC, EC, PVP, and PVA.

Introduction

Since long back transdermal drug delivery system made an increased interest as controlled release drug delivery system for chronic manageable diseases like hypertension, asthma, diabetes etc to reduce frequency of dosing. It increases the safety profile of highly potent drug and improves patient compliance. Glipizide is a potent drug so formulated as transdermal drug delivery system to maintain plasma drug concentration within therapeutic range. [1]

A transdermal patch or skin patch is a medicated adhesive patch that is placed on the skin to deliver a specific dose of medication through the skin and into the blood stream. The transdermal route of administration is recognized as one of the potential route for the local and systemic delivery of drugs. Transdermal route has advantages over conventional modes of drug administration as it avoids hepatic first pass metabolism and improve patient compliance. However the highly organized structure of stratum cornium forms an effective barrier to the permeation of drugs, which most be modified if poorly penetrating drugs are to be administered. [2,3] The use of chemical permeation enhancers would significantly increase the permeation of released drug from polymer matrix. A transdermal patch has several components like liners, adhesives, drug reservoirs, drug release membrane etc, which play a vital role in the release of drug via skin. Because of its great advantages it has become one of the highly research field among the various novel drug delivery system. [4, 5]

The matrix diffusion controlled transdermal patches can be formulated by using different types of polymers like HPMC, EC, PVP, PVA and various natural polymers like Guargum, xanthum gum, cellulose etc. [6,7]

Hydrophilic polymers like HPMC is widely used for designing controlled release drug delivery system because of their flexibility to provide a desirable drug release profile, cost effective and compatible with drug molecules and other excipients. But use of hydrophilic polymer alone causes rapid release of drug and has uncontrolled rate of release. To overcome this problem, hydrophobic polymers are included in matrix system. Keeping in this view, the present investigation is aimed at designing suitable release matrix diffusion controlled TDDS using polymers like HPMC K15M, EC, PVA and polyvinyl pyrrolidone (PVP). [8, 9, 10]

Glipizide is a second generation sulfonyl urea, compound that is used as a hypoglycemic agent and is commonly prescribed for the treatment of patient with type –II diabetes. Glipizide stimulates insulin secretion from the cells of pancreatic vein and may increases the concentration of insulin in pancreatic vein and may increase the number of insulin receptors. Glipizide is a weak acid (P^{ka} = 5.9), practically insoluble in water and in acidic environment and highly permeable (BCS class–II) drug. The oral absorption is uniform, rapid and complete with a bioavailability of nearly 90% and an elimination half life of 2-4 hrs. As Glipizide has a short half life, is administered orally in 2 to 3 divided dose of 2.5 to 10 mg per day. In the present study Glipizide is formulated as transdermal drug delivery system for release of the drug up to 24 hrs. [11, 12, 13]

Material and Methods

Materials

Glipizide BP was procured as gift sample from Suneshine lab Ltd with batch no 010707/GP, assay-99.4%, LOD- 0.14% w/w. HPMC K15M, EC, PVP K30, PVA were purchased from SD fine chemicals, Mumbai. Acetone and other solvent were of analytical grade.

Methods

Formulation of matrix controlled Glipizide patches:

Matrix type transdermal patches loaded with Glipizide were prepared by solvent casting method. Required quantities of polymers were weighed and dissolved in 20 ml of acetone, sonicated for 30 minute and then stirred for one hr in a magnetic stirrer at 400 rpm. 147.78 mg of drug was weighed and added to the above solution. Required quantity of PEG 4000 (as plasticizer) and propylene glycol (as permeation enhancer) were measured and added to the above solution. The above mixture is then stirred in a magnetic stirred at 400 rpm for 2 hrs. The resulted uniform solution was cast on a petridish of area of 66.50 cm^2, previously containing a layer of mercury. An inverted funnel was placed over the Petri dish to prevent the fast evaporation of the solvent. After 24 hrs the dried patches were taken out, cut into pieces of 3 cm×3 cm (area=9 cm^2 and containing 20 mg of the Glipizide) and stored in a desiccator. The compositions of different batches are shown in table-1.

Evaluation of Glipizide transdermal patches for different formulations [11, 12, 13]

Drug polymer compatibility study

Drug-polymer compatibility study was performed by comparing FTIR spectra of optimized formulation F_8 with drug Glipizide and excipients like HPMC K15M, PVP K30, PVA and EC etc. The FTIR studies were carried out at SIPRA LAB., Hyderabad, India.

Thermal Analysis

To ensure the thermal stability of formulation, DSC (differential Scanning Calorimetry) was performed for pure drug Glipizide as well as with optimized formulation (F_8). For DSC study, the sample were heated from sub-zero temperature to 300 ^{0} C at a heating rate of 10 ^{0}C/min under nitrogen atmosphere in aluminum foil. The DSC studies were carried out at SIPRA LAB., Hyderabad, India.

Table-01

Composition of different formulations of Glipizide transdermal patches

Sl. No.	Name of the ingredients	F_1 (mg)	F_2 (mg)	F_3 (mg)	F_4 (mg)	F_5 (mg)	F_6 (mg)	F_7 (mg)	F_8 (mg)
1	Glipizide	147.78	147.78	147.78	147.78	147.78	147.78	147.78	147.78
2	HPMC K15M	400	300	600	500	600	800	600	600
3	EC	600	700	400	500	300	200	300	300
4	PVP K30	100	100	100	100	200	100	-	100
5	PVA	-	-	-	-	-	-	200	100
6	PEG 4000	200	200	200	200	200	200	200	200
7	DMSO	10	10	10	10	10	10	10	10
8	TiO_2	10	10	10	10	10	10	10	10
10	Acetone	20	20	20	20	20	20	20	20

Thickness of patch

The thickness of the patch is measured by digital micrometer at different points. For each formulation three patches were used. The average value for the thickness of single patch was determined. The results are shown in table.

Weight uniformity

The prepared patches were dried at 60 ^{0}C for 4 hrs before testing. Weight uniformity was done by weighing 5 different patches of each batch. All the patches selected at random should uniform in size (3 cm × 3 cm). The average weight of three is calculated. The results are shown in table.

Folding endurance

A strip of specific area (3 cm × 3 cm) was cut evenly and repeatedly and folded at the same place till it broke. The number of times the film could be folded without breaking gave the value of folding endurance. The results are shown in table.

Percentage moisture content

There patches of the same composition were weighed and kept on desiccators containing fused calcium chloride at 37 ^{0}C until no change in weight of the individual patches was observed. This weight was noted as the final weight. The percentage moisture content was calculated as a difference between individual and final weight. The results are shown in table-

Percentage elongation break test

The percentage elongation is determined by measuring the length just breaking point. The percentage elongation can be determined from the below mentioned formula.

% Elongation = $(L2 - L_1)/L_1 \times 100$

Where L_1 = Initial length of each strip

L_2 = Final length of each strip

Percentage Drug content

The patch of 2 cm^2 was cut into small pieces and taken into 100 ml volumetric flask containing 20 ml of methanol. This methanolic solution was diluted with phosphate buffer P^H 7.4 up to 100 ml & the solution was filtered through whatman filter paper & the drug content was determined on spectrophotometer at λ_{max} 274 nm after suitable dilutions.

In- vitro drug release studies

The in- vitro drug release studies were carried out in a specifically designed Franz diffusion cell. The cellulose acetate membrane of pore size of 0.45 µm was mounted between donor and the receptor compartment of diffusion cell. The transdermal film was placed on the cellulose acetate membrane and covered with aluminium foil. The receptor compartment was filled with freshly prepared phosphate buffer P^H 7.4 (550 ml). The whole assembly was fixed on a magnetic stirrer with hot plate apparatus. The solution in receptor compartment was stirred at 30-40 rpm and temperature was maintained at 32 ± 0.5 ^{0}C. 3ml of sample was withdrawn at different interval and replaced with the same volume of the phosphate buffer P^H 7.4. Samples were analysed spectrophotometrically at λ_{max} 274 nm.

Kinetic release profile: [15, 16]

To study the release kinetics, data obtained from in vitro drug release studies were plotted in various kinetic models. Zero order kinetic was plotted as cumulative percentage amount of drug release vs. time.

$$C = K_0 t$$

where C = Cumulative drug release at time t.

K_0 = Zero order rate constant

First order kinetic was plotted as log cumulative percentage amount of drug release vs. time.

$$log\ C = log\ C_0 - \frac{Kt}{2.303}$$

where C = Amount of drug remained at time t

C_O = Initial amount of drug

K = First order rate constant

Higuchi kinetic was plotted as cumulative percentage amount of drug release vs. square root of time.

$$Q = K\ t^{1/2}$$

Q = Cumulative percentage drug release at time t.

Korse-Meyer Peppas equation: $F = Mt/ Má = K_m t_n$

Where M_t is the drug released at time t, $M_á$ is the total amount of drug in dosage form, F is the fraction of drug release at time t, Km is a constant dependant on geometry of dosage form, n is the diffusion exponent indicating mechanism of drug release.

Skin irritation study for formulation F_8

Skin irritation study was performed on the healthy Albino rabbits weighing between 1.5-2.5 kg from which the dorsal hair is removed and it was cleaned using rectified sprit. Aqueous solution of formalin 0.8% was used as a standard irritant. Prepared transdermal patches of area 15 cm^2 were used as a test patch. 0.8% of formalin solution was applied in the right dorsal surface of each rabbit whereas test patches were applied on the left dorsal surface. The patches were removed after 24 hrs and skin was examined.

Kinetic release profile [15, 16, 17, 18]

To study the release kinetics, data obtained from in vitro drug release studies were plotted in various kinetic

models. Zero order kinetic was plotted as cumulative percentage amount of drug release vs. time.

$C = K_0 t$

where C = Cumulative drug release at time t.

K_0 = Zero order rate constant

First order kinetic was plotted as log cumulative percentage amount of drug release vs. time.

$log\ C = log\ C_0 -$

where C = Cumulative drug release at time t.

C_O = initial drug concentration

K = first order rate constant

Higuchi kinetic was plotted as cumulative percentage amount of drug release vs. square root of time.

$Q = K\ t^{1/2}$

Q = Cumulative percentage drug release at time t.

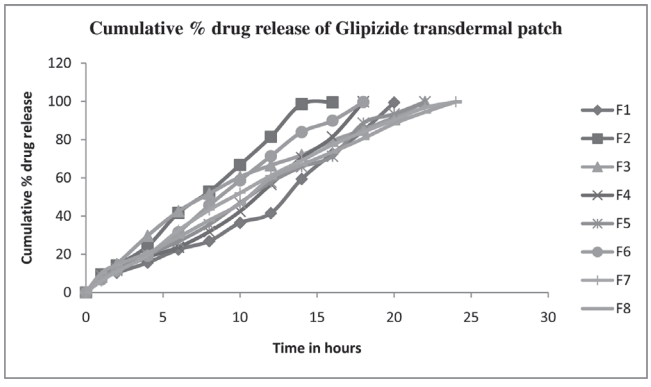


Fig 1: Graph showing in vitro release study of formulation $F_1 - F_8$

Results and Discussion

Drug- polymer compatibility study

The FTIR spectra of Glipizide and patch shows that a sharp peak is obtained at 3424 cm^{-1} to 3423 cm^{-1} indicating presence of intermolecular OH^- group. Comparing FTIR spectra of HPMC K15M and patch shows that a sharp peak is observed at 3443 cm^{-1} to 3423 cm^{-1} indicating presence of intermolecular broad OH^- stretching. Comparing FTIR spectra between PVA and patch shows that a sharp peak is observed at 3324 cm^{-1} to 3423 cm^{-1} indicating presence of same functional group. Between PEG 4000 & patch the sharp peak is observed at 3441 cm^{-1} to 3423 cm^{-1} that indicates the functional group present in PEG 4000 that also appears in patch and between EC and patch , the sharp peaks are observed at 3454 cm^{-1} and 3423 cm^{-1} respectively which indicates that the functional group that appears wit EC also appears at patch. So FTIR study indicates that in all ingredients, the peaks is observed between 3420 cm^{-1} to 3443 cm^{-1} and one broad peaks is observed between 3420 cm^{-1} to 3445 cm^{-1} which indicates that there is no interaction between polymer HPMC K150M, EC, PVP, PVA, PEG 4000 and Glipizide. The FTIR data are given in figure-3.

Thermal analysis

DSC thermogram of Glipizide and patch were observed that the endothermic peak appeared at 213.7 ^0C and 231.3 ^0C respectively which indicates that the patch is thermodynamically stable because the patch required more heat than pure drug due to presence of various polymer like HPMC K15M, EC, PVP AND PEG 4000. The DSC data are given in figure-2.

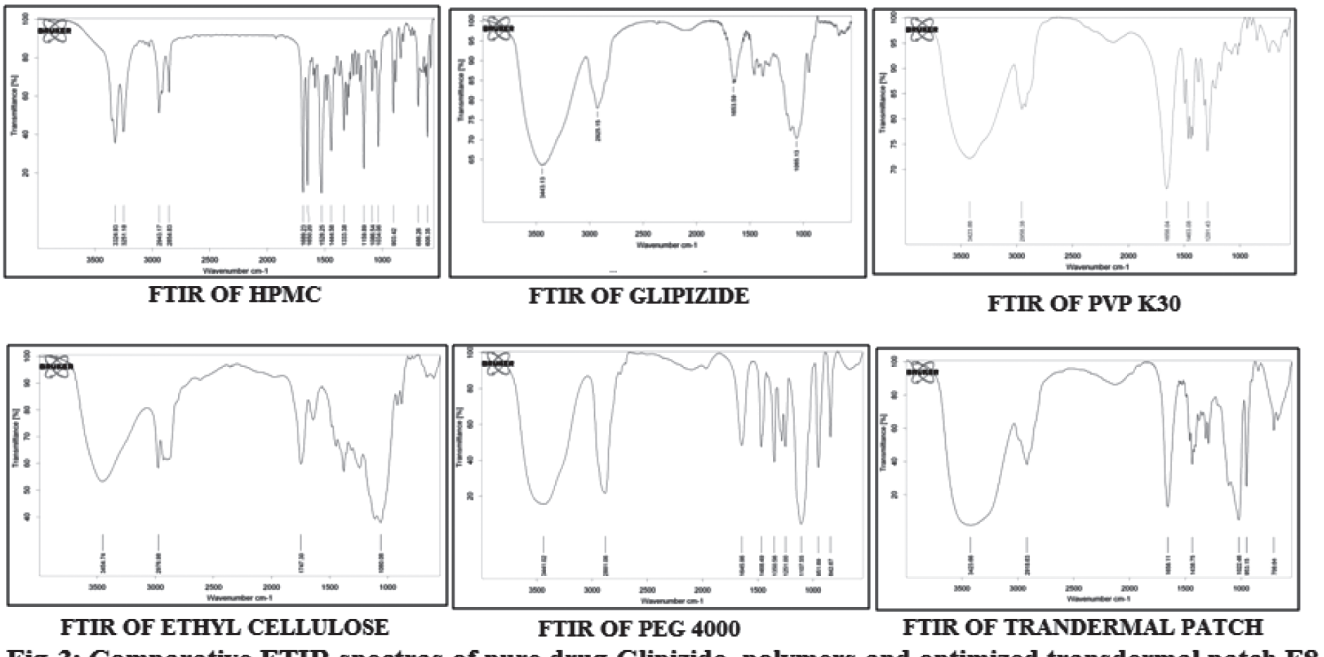


Fig-3: Comparative FTIR spectras of pure drug Glipizide, polymers and optimized transdermal patch F8

Table-2

Evaluation Results of various physicochemical parameters

Formulations	Weight variation mean (mg)±SD	Thickness Mean (mm)±SD	Moisture content (Weight %)	Folding endurance	% elongation break test	% Drug content
F_1	312±3.13	0.16±0.012	1.15	168±5	41.2	99.44
F_2	316±2.08	0.18±0.017	1.02	178±6	36.6	98.89
F_3	324±3.09	0.20±0.013	1.84	197±4	38.8	96.48
F_4	308±4.15	0.17±0.012	1.64	179±3	46.9	99.64
F_5	318±1.49	0.19±0.015	1.88	217±4	51.4	99.71
F_6	317±2.19	0.21±0.018	1.96	210±6	42.5	97.97
F_7	318±1.02	0.20±0.017	1.78	212±5	49.2	98.49
F_8	319±1.31	0.19±0.014	1.91	218±4	50.7	99.69

The physicochemical evaluation of transdermal patches

The results of the physiochemical evaluation of the transdermal patches are given in table 2. The weight variation of all the formulations varied in between 308±4.14 mg to 318±1.49 mg. The variation of thickness of all the formulation was in the range of 0.16±0.012 mm to 0.21±0.018 mm. Moisture content of different formulation was found to be in the range of 1.02 to 1.96 %. The difference in the moisture content may be due to differences in hydrophilicity of the polymers and extent of solvent evaporation during formulation. Folding endurance was to be in between 168±5 to 217±4. The % elongation break test was found to be in between 35.4 to 51.4%. The folding endurance and % elongation break test was found maximum in formulation containing more amount of PVP K30 as polymers. The % drug content was found to be maximum (99.48%) in formulation F_4.

In vitro release study

In vitro release study for all the prepared patches of different formulation were carried out for 24 hrs. % cumulative drug release after 24 hrs was taken and compared for all the patches. Formulation F_8 exhibit drug release upto 24 hr and in a controlled rates then other formulations. F_2 release approximately 99.49% in 16 hrs which released drug to least period of time then other formulations. The results are shown in table-3.

Skin irritation study

The skin irritation study was carried out with optimized formulation F_5 on Albino rabbits. No sign of erythema or redness were observed for 24 hrs after the application of the patch which was compared with standard 0.8% formalin solution.

Table 3

Cumulative percentage drug release study results of different formulations

Time (Hour)	F_1	F_2	F_3	F_4	F_5	F_6	F_7	F_8
1	7.18	9.48	8.33	9.66	08.89	6.84	6.09	10.19
2	10.29	14.19	14.81	11.65	12.41	11.49	10.86	14.48
4	15.63	24.28	29.68	18.41	19.65	19.16	19.58	20.67
6	22.48	41.69	42.48	23.75	26.71	31.48	32.49	28.94
8	26.87	52.78	51.74	31.85	35.52	45.63	43.23	37.81
10	36.47	66.82	60.49	42.47	46.91	58.66	52.09	46.49
12	41.52	81.49	66.59	56.61	57.74	71.39	61.16	59.84
14	59.41	98.61	71.94	70.54	65.80	83.94	69.12	66.80
16	72.69	99.49	79.43	81.46	71.32	89.94	77.33	73.52
18	84.73	-	85.24	99.83	88.61	99.66	84.05	80.64
20	99.46	-	91.71	-	93.41	-	90.28	88.37
22	-	-	99.68	-	99.78	-	96.73	94.42
24	-	-	-	-	-	-	99.79	99.81

***In-vitro* kinetic study**

In-vitro released data for optimized formulation (F_8) was tested for zero order, first order, korse-Meyer Peppas model and Higuchi model. The slope value and regression coefficient was calculated for each model and given in table-5.

Discussion

Glipizide is an oral hypoglycemic agent which is a commonly prescribed drug for treatment of patients with type-II diabetes (non insulin dependent). Diabetes mellitus is chronic disease and requires constant level of insulin that can be achieved by constant and uniform supply of drug for extended period of time.

FTIR and DSC studies revealed that the drug and polymer were compatible with each other and formulation is thermally stable. Of all the formulation prepared and evaluated, F_{-5} and F_8 showed promising physicochemical characteristics like weight uniformity, thickness of patch, folding endurance, percentage moisture content, percentage elongation break test, drug content etc. The in vitro drug diffusion study of F_8 formulation was found to be best among other formulation due to initial burst release within 1 hr followed by better controlled release of drug up to 24 hrs may be due to presence of equal proportion of PVP K30 and PVA. So the formulation F_8 shows initial release of loading dose followed by controlled release up to 24 hours will have better therapeutics benefits. The reason behind better controlling effect of F_5 formulation is due to more % of HPMC K 15M and PVP then other formulation may be because of hydrophilic nature of polymer, as drug is hydrophobic in nature but it released all most all the drugs within 22 hours. The release rate of F_2 formulation

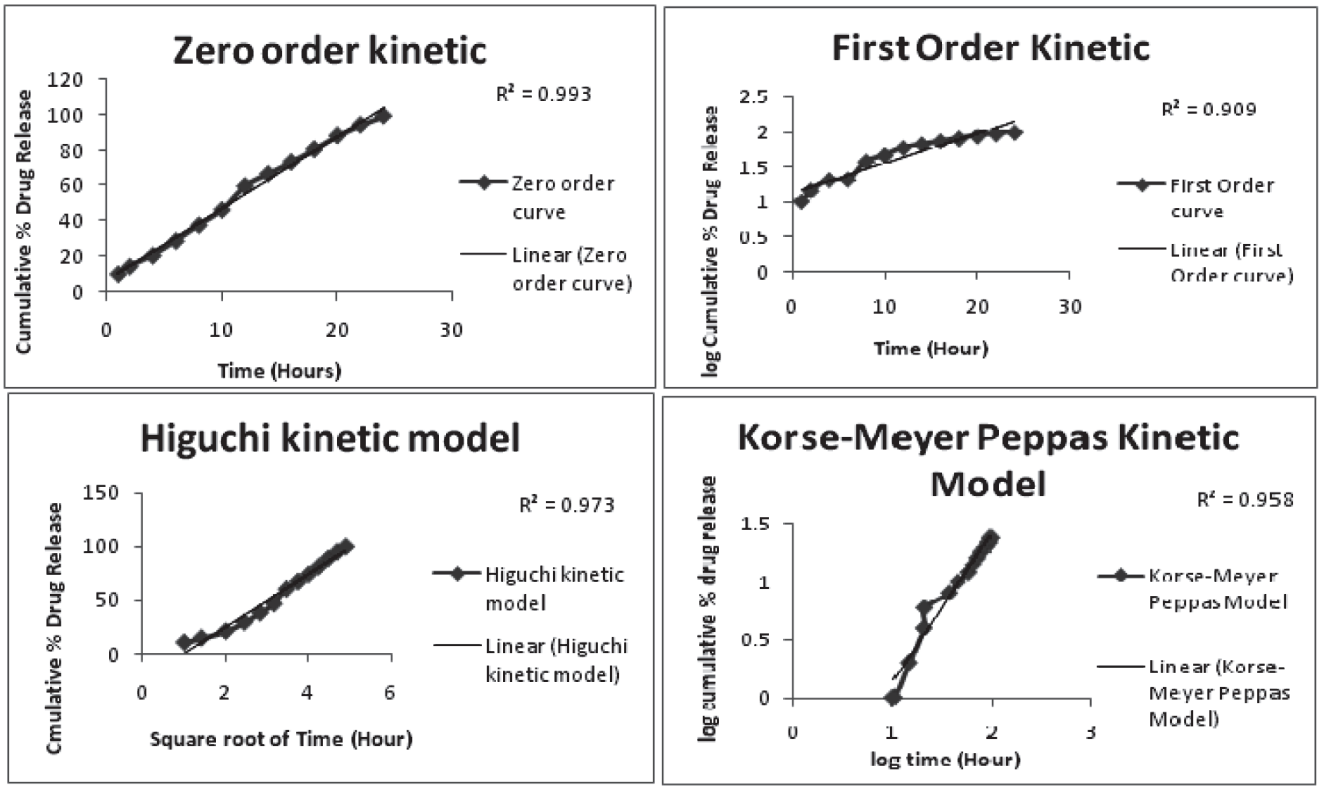


Fig-4: In vitro release kinetic study of optimized Transdermal patch formulation (F_8)

Table-5
In-vitro release kinetic study of optimized Transdermal patch formulation (f_8)

Formulation	Regression coefficient of Zero order	Regression oefficient of 1st order	Regression coefficient of Higuchi model	Regression coefficient of Korse-Meyer Peppas model	Conclusion
Optimized Glipizide Transdermal Patch (F_8)	0.993	0.909	0.973	0.958	Release kinetics follows zero order

was low due to presence of more percentage of EC and both are hydrophobic in nature. Formulations F_7 contain only PVA without PVP K30 and show better controlled release effect than other formulations but initial drug release within one hour is low, that may not be sufficient to elicit pharmacological response. From in vitro release kinetic study it was concluded that the release kinetic follows zero order kinetic because the regression value for zero order kinetic is maximum i.e 0.993 then other kinetics like first order, Higuchi model, and Korse-Mayer peppas model.

Conclusion

From the results and discussion among the 8 formulations designated as F_1, F_2, F_3, F_4, F_5, F_6, F_7 and F_8; the formulation F_8 was found to be successful in terms of controlled release with longer period of time and maximum percentage of drug release containing HPMC K15M, EC, PVP K30, and PVA having ratio of 6**:**3:1:1 respectively. The prepared optimized formulation can fulfill patient requirements and can reduce frequency of dosing. This formulation transdermal patches, may increase the bioavailability of Glipizide as it by pass fast pass metabolism so utilization of maximum amount of drug can be achieved.

Acknowledgement

Author thanks to Sunshine laboratories limited (Hyderabad, India) to provide Glipizide BP pure drug as a gift sample. Thanks to SD fine chemicals, Mumbai for providing HPMC K 15M, EC, PVP K30, PVA and lactose. Author is also thankful to management and principal Anwarul Uloom College of Pharmacy, NewMallepally, Hyderabad for providing laboratories and reagents for research work. Thanks to library staff Anwarul Uloom College of Pharmacy, NewMallepally, Hyderabad for providing the facility and their active support to carry out literature survey.

References

1. Front line strategic consulting Inc. Alternative Drug delivery system series: Transdermal drug delivery systems. 2002.
2. Anon. Transdermal delivery systems- general drug release standards. Pharmacopoeia forum, 1980, 14 (1), 108-110.
3. Cross, S. E.; Roberts, M. S.; current Drug Delivery, 2004, I, 81-92
4. Gwen MJ., Joseph RR., Banker GS and Rhodes CT, (editors). Modern pharmaceutics. 3rd ed., Morcel Dekker. Inc, 1996, 575.
5. Chein YW., Novel drug delivery system. 2nd ed. Morcel Dekker. Inc, 1997, 1-42.
6. www. Medicines.org.uk/emc/browsedocuments
7. Aulton ME. Wells TI., Pharmaceutics: The Science of Dosage Form Design, London, England: Churchill Livingstone: 1988: 185-189.
8. Goodman and Gillman's, The Pharmacological Basis of Therapeutics, 11th ed. Laurence L. Brunton, John S. Lazo, 1635-1638.
9. N. K. Jain, Controlled and Novel Drug Delivery; 2008 ed. Page no-100-130.
10. S.P. Vyas, R.K. Khar, Controlled Drug Delivery concepts and Advances; 2006 ed. 411-448.
11. Berner B, John V A Pharmacokinetic characterisation of Transdermal delivery systems Jour. Clinical pharmacokinetic, 1994, 26(2), 121-134.
12. Indian Pharmacopoeia. 4th Edn., Vol-II., Controller of Publication., Govt.of India., New Delhi.,1996, 735.
13. www.drugbank.com
14. Rao PR, Ramakrisna S, Diwan PV,; Drug release kinetics from polymeric films containing propranolol hydrochloride for transdermal use, 2000, 5(4), 465-472.
15. P. Chirag J. et al; formulation and evaluation of matrix diffusion controlled transdermal drug delivery system; jour. Of drug delivery and therapeutics: 2012, 2(1)
16. Heather A.E. Benson, Transdermal Drug Delivery: Penetration Enhancement Techniques; Current Drug Delivery, 2005, 2, 23-33.
17. Sood A, Panchagnula R, Role of dissolution studies in controlled release drug delivery system. STP Pharma Sci. 1999, 9(2), 157-168
18. Hadjioannu TP et.al, Quantitative Calculations In Pharmaceutical Practice and Research, New York: VCH Publishers Inc., 1993, 345-348.

ISSN 0973 – 9874

Formulation & Evaluation of Controlled Drug Delivery System for Anti-asthamatic Drug Using Cross Linking Technique

C.M. PRASAD[1] AND O.P. THAKUR[2]*

[1]Retd. Professor, Department of Pharmaceutical Sciences, BIT Mesra, Ranchi, Jharkhand
[2]*Assistant Professor, Department of Pharmacy,MIT Muzaffarpur, Bihar

ABSTRACT

The aim of this study was to formulate & evaluate microencapsulated Controlled release preparation of an Antiasthamatic drug Salbutamol Sulphate using gelatin with cross linking technique. Different amounts of gelatin were added in order to obtain various drugs to polymer ratio. Two different methods of cross linking were adopted. Microcapsules were classified with respect to particle size distribution. The products were evaluated with respect to encapsulation efficiency, drug content & in-vitro release pattern using USP dissolution apparatus.

Keywords: Salbutamol Sulphate, Cross linking technique, Microcapsules.

Introduction

Salbutamol belongs to Sympathomimetic amines having receptor activity (both ²1 & ²2) which can bring about bronchodilation following per oral administration[1]. It has prolonged distribution phase lasting 1-2 hours, terminal half-life in the range of 3-8 hours & peak serum concentration of approximately 3 hours. There appears to be incomplete bio-availability with only 70% of administered radiolabel appearing in the urine [2]. Salbutamol sulphate is fairly soluble in water & therefore the construction of a sustained release product in microcapsules is more convenient. This gets distributed throughout the gastrointestinal tract, improves drug absorption, reduces side effects related to localized build up of irritating drugs against the gastrointestinal mucosa, improves efficiency of dosage regimen by providing an initial burst of drug to facilitate rapid onset of action & then maintain a constant plasma drug level for a prolong period of time [2-5].

In the formulation of microcapsules, the drug is dispersed in the gelatin solution for providing enormously large surface area & then this gelatin is poured in a hydrophobic medium with continuous stirring. The strong cohesive force of gelatin solution facilitates the formation of different sizes of spherical micropellets depending upon temperature, viscosity of the medium & the ratio of drug gelatin in the mixture. Further, cross linking the micropellets with formaldehyde results in the formation of retarded solubility in gastric juice for further sustaining the action of drug [6].

The purpose of this investigation was to prepare Salbutamol Sulphate microcapsules using gelatin as wall material.

MATERIALS AND METHODS

Materials

Salbutamol Sulphate,Gelatin: Bloom strength 277gms, Iso-electric pH 8.7(40^0),Liquid Paraffin I.P. Viscosity - 97.7108 centistokes at 37^0,Light liquid Paraffin I.P. Viscosity -16.8315 centistokes at 37^0 Formaldehyde A.R. 40%, Isopropyl Alcohol, Molecular Weight 60.10, Boiling range 81-83.5^{0}C.

Methods

Characterization of bulk drug & analytical monitoring

Bulk drug was characterized by various official tests of identification[7]&analyzed by UV Spectrophotometric method. 278nm & 296nm were used for analytical monitoring of products in 0.1N HCl & 0.1N NaOH solution respectively.

Preparation of drug delivery system

The formulations were prepared in the proportions of 1:3, 1:2, 1:1 as the drug to gelatin ratio by dispersing sufficient amount of drug of 100 mesh size into warm (50^0-60^0C) aqueous gelatin solution with the help of electric stirrer which was poured in this & steady stream into warm (55-60^{0}C) mixture of liquid paraffin of absolute viscosity

*Address for correspondence
E-mail:thakur.op18@gmail.com

of 23.92 Cp at 55^0 with uniform stirring rate 200-250 r.p.m. Then quickly cooled to get gelatin micro drops followed by addition of chilled isopropanol, pellets were segregated & stored in refrigerator overnight. It was then dried in vacuum desiccators for 24 hours. The product was then exposed to previously saturated vapour of 40% w/w formaldehyde solution in a desiccator for variable times. After stipulated period of exposure micropellets were dried in warm air for cross linking. Another method of cross linking was adopted by addition of varying percentage of formaldehyde to micropellets & keeping in contact for 12 hours.

Evaluation of Drug Delivery System

Determination of encapsulation efficiency:

100mg of uniformly pulverized micropellets is dissolved in 0.01N HCl by subjecting it to magnetic stirring for 2 hours. The liquid was filtered off using Whartmen filter paper. An aliquot of this was treated as earlier for determination of drug content against blank placebo of gelatin micropellets treated & then its UV absorption was noted.

Particle Size analysis:

Particle size distribution was determined using Standard Sieve Shaker with sieves having double gyratory & vibratory method.

Dissolution Rate Study:

This study was conducted using dissolution apparatus[8] with the basket covered with 100 mesh nylon, screen bonded permanently to the inner wall. Dissolution studies were carried out at the designated pH profile which has been reported to simulate gastrointestinal pH conditions [9]. The pH profile was achieved using the solution of hydrochloric acid, sodium carbonate & sodium hydrogen carbonate in double distilled water. The apparatus is adjusted to 50 r.p.m. speed & with 37+/-0.5p C temperature of the dissolution media. Samples were taken at appropriate intervals up to 8 hours & then filtered. Salbutamol content was determined spectrophotometrically as reported earlier. Each release determination was carried in triplicate.

RESULTS & DISCUSSION

Encapsulation efficiency of microcapsules with 1:3 ratio is 33.09+/-3.620, with 1:2 ratio is 61.60+/-4.934 & with 1:1 ratio is 40.12+/-6.508. In this case, 1:2 ratio shows good encapsulation efficiency than 1:3 & 1:1 ratio. So, it can be considered as an optimum ratio.

Content of drugs in the micropellets varied depending on the method of rigidization. Due to variable intrinsic solubility of Salbutamol Sulphate, some amount of drug is lost in the manufacturing vehicles depending on the process of rigidization. Significant amount of drug was lost in situ rigidization process which may be due to drug leaching as well as diffusion (Table 1).

The optimum particle size was chosen 32/44 mesh i.e. 350-450 micrometer because this size is more suitable for capsule filling due to its uniform flow ability for dissolution rate studies. It is observed that release of drugs is affected by drug-gelatin ratio. The optimum drug-gelatin ratio was found to be 1:3. This proportion gave maximum release of the drug within a specified period as shown in fig 1. The drug release rate is also affected by particle size which has been represented in fig 2. It was observed that larger particle of 20/32 mesh failed to release the total drug within the scheduled period of experiment. The drug release rate increased with the decreased particle size. Diffusion path length is found to be less in case of smaller particles. The total release rate at the end of the study & individual release rate have been observed higher in high drug-gelatin ratio.

Rigidization is dependent on time of exposure of formaldehyde vapour with micropellets. The process of cross linking is slow because surface of micropellets do not get uniform exposure to the vapour, which produces uneven rigidized surface which results in formation of channels to leach out drug as evident from fig 3. Method of rigidization does affect release rate of the drug which is evident with the observation of fig 4. This method provides higher release rate of the drug because the cross linking of gelatin occurs while the matrix is soft. During the drying process, the water is evaporated forming channels in the matrix which makes it Swellable in contact with water. Thus the drug is much easily diffused from the spongy matrix surface.

Table 1
Pharmaceutical Properties of Salbutamol Sulphate Micropellets

Rigidizing Agent	Parameter	Method of Rigidization					
		Formaldehyde Vapour Duration of rigidization (hrs)			In Situ Concentration of Agent		
		48	72	92	3%	5%	10%
Formaldehyde	Yield%	84.76	84.53	85.79	93.72	94.22	92.75
	Content Uniformity	84.98	85.39	84.25	77.49	76.15	75.05

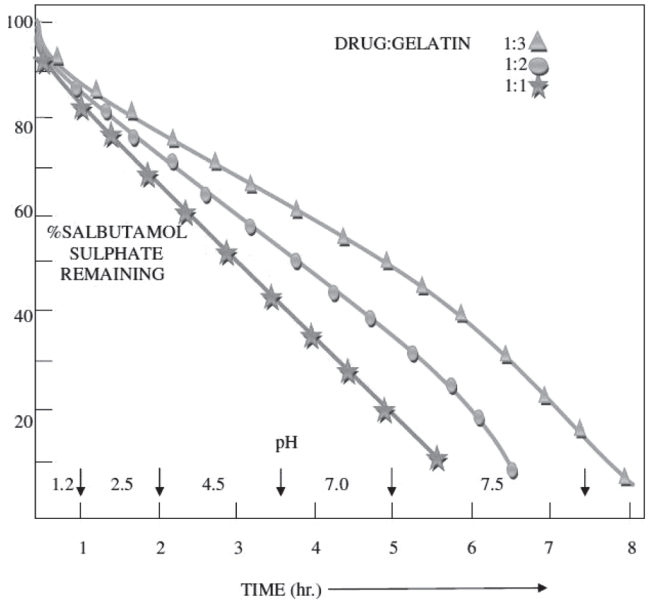


Fig.1 : Effect of Drug: gelatin ratio of Micropellets on in vitro dissolution profile of Salbutamol Sulphate; sieve size 32/44, rigidizing agent Formaldehyde Vapour for 72 hrs.

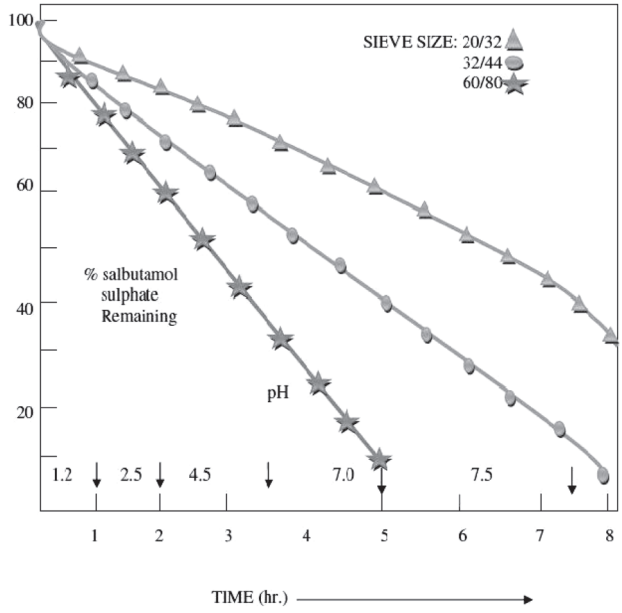


Fig.2: Effect of particle size of Micropellets in vitro dissolution profile of Salbutamol Sulphate; sieve size 32/44, rigidizing agent Formaldehyde vapour for 72 hrs.

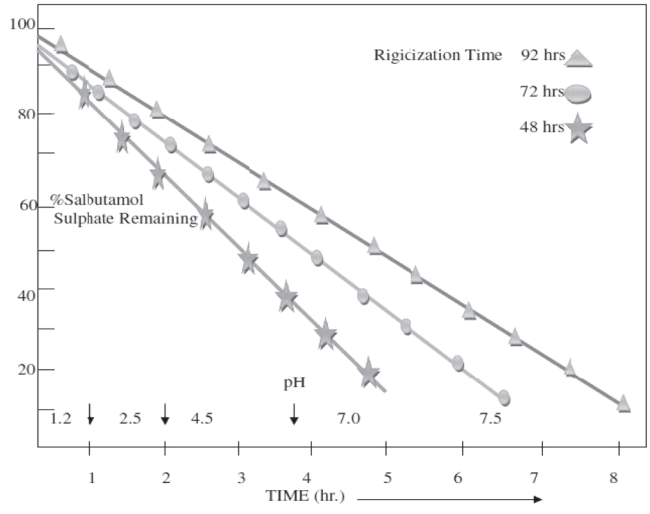


Fig.3: Effect of Formaldehyde Vapour Rigidization of Micropellets on in vitro dissolution Profile of Salbutamol Sulphate; sieve size 32/44, drug: gelatin ratio 1:2.

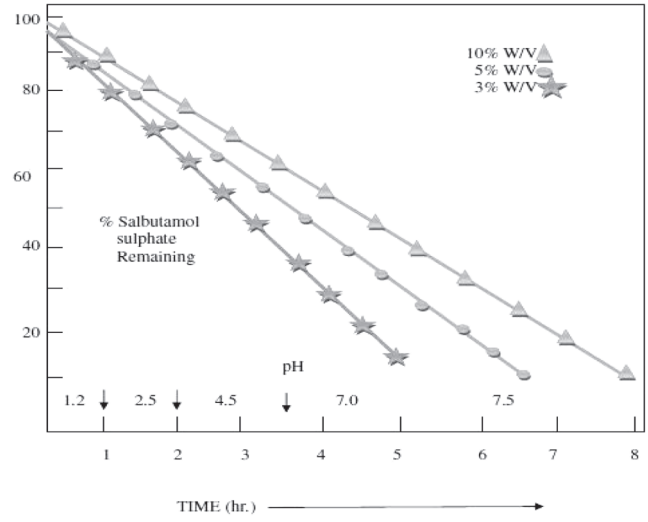


Fig.4: Effect of in situ Rigidization of Micropellets with formaldehyde on in vitro dissolution profile of Salbutamol Sulphate; sieve size 32/44, drug: gelatin ratio 1:2.

REFERENCES

1. Gerdikas, K. & Papadatos, K., Greek Recipe Book, Greek National Organization of Drugs, Athens, 1987, PP. 71-73.
2. *Aqil M, Ali A, Sultana Y, Najmi AK. Pharmazie. 2004 Aug; 59(8):631-5.*
3. *Dixit RB, Uplana RA, Patel VA, Dixit BC, Patel TS. Sci Pharm. 2010; 78(4):909-25.*
4. Evans, M.E., Walker S.R., Brittain, R.T. & Peterson, J.W., Xanobiotica, 3, 1973, PP 113.
5. Li, S.P., Kowalski, C.R., Field, K.M. & Grim, W.M., Recent Advances in Microencapsulation Technology & Equipment, Drug development industry pharmacy, 14(1988) 353-376.
6. Tanoka, N., Takino, S. & Utsumi, I., J. Pharmaceutical Sciences, 52, 1963, PP 664.
7. Indian pharmacopoeia, Ministry of health & family welfare, Govt of india, Neo Delhi 1996.
8. US Pharmacopoeia, XXIst Edu.,Mack, Easton, PA 1985, PP 630-651.
9. Takenaka H, Kawashime Y, Lin S.Y., J.Pharma. Science, 69, 1980, PP1388.

ISSN 0973 – 9874

Evaluation of Antioxidant Activities of Aqueous Extract of Stem Bark of *Boswellia Ovalifoliolata* in Streptozotocin Induced Diabetic Rats

Y.K. PRABHAKAR[1], MD. SUBHAN ALI[1], M.V. JYOTHI KUMAR[2], T. KRISHNA TILAK[1] AND CH. APPA RAO[1*]

[1]Department of Biochemistry, Sri Venkateswara University, Tirupati, AP, India.
[2]Department of Biotechnology, Sri Venkateswara University, Tirupati, AP, India.

ABSTRACT

This study was designed to investigate the antioxidant activity of the aqueous extract of stem bark of *Boswellia ovalifoliolata* (AESBBO) in streptozotocin (STZ) induced diabetic rats. Oral administration of aqueous extract at a dose of 200 mg /kg bw/day/ for 40 days significantly decreased hepatic and renal thiobarbituric acid reactive substances (TBARS) and activity of catalase (CAT). There was a significant improvement in the activities of superoxide dismutase (SOD), glutathione peroxidise (GPx) and glutathione-s-transferase (GST) in liver and kidney of STZ induced diabetic rats after treatment with AESBBO when compared with untreated diabetic rats. These results clearly indicate that aqueous extract of *Boswellia ovalifoliolata* (AESBBO) possess significant antioxidant effect in diabetic rats.

Keywords: Antioxidant activity, *Boswellia ovalifoliolata*, Glibenclamide, Streptozotocin.

Introduction

Herbal medicines are the oldest remedies known to mankind. In the present scenario, the demand for herbal products is growing exponentially throughout the world and major pharmaceutical companies are currently conducting extensive research on plant materials for their potential medicinal value. In many journals, national and international, we find an increasing number of research publications based on herbal drugs. Many analysis-based studies regarding pharmacological research in India have been conducted in the past [1].

Diabetes is a metabolic disorder characterized by hyper glycemia resulting due to deficiency of insulin secretion by pancreas, ineffectiveness of produced insulin, or both [2]. It is the most important non-infective epidemic to hit the globe in the present millennium. The number of people suffering from diabetes worldwide is increasing at an alarming rate. It is predicated that about 366 million people are likely to be diabetic by the year 2030 [3]. It causes number of complications like retinopathy, neuropathy, and peripheral vascular insufficiencies [4]. Hyperglycemia can be handled initially with oral synthetic agents and insulin therapy. However, these synthetic agents produce some serious side effects and are relatively expensive for developing countries [5]. The toxicity of oral antidiabetic agents differs widely in clinical manifestations, severity, and treatment [6]. In the natural system of medicine many plants have been claimed to be useful for the treatment of diabetes mellitus. The dependence of large rural population on medicinal plants for treatment of diabetes is because of its availability and affordability [7]. In recent years, several authors evaluated and identified the antidiabetic potential of traditionally used Indian medicinal plants using experimental animals. Although a large number of medicinal plants have been tested for their antidiabetic effects, it remains to be investigated in several other Indian medicinal plants.

Excessive oxidative stress is observed in the diabetes [8]. Oxidative stress is currently suggested as mechanism underlying diabetes and diabetic complication [9]. During diabetes, persistent hyperglycemia causes increased production of free radicals, especially reactive oxygen species (ROS) for all tissues from glucose auto-oxidation and protein glycosylation. The increase in the level of ROS in diabetes could be due to their increased production and or decreased destruction by non enzymatic and enzymatic antioxidants. The level of these antioxidants critically influences the susceptibility of various tissues to oxidative stress and is associated with the development of complications in diabetes [10]. Oxidants are generated as a result of normal intracellular metabolism in mitochondria and peroxisomes, as well as from a variety of cytosolic enzyme systems. In addition, a number of external agents

can trigger ROS production. A sophisticated enzymatic and nonenzymatic antioxidant defence system including catalase (CAT), superoxide dismutase (SOD) and reduced glutathione (GSH) counteracts and regulates overall ROS levels to maintain physiological homeostasis. Lowering ROS levels below the homeostatic set point may interrupt the physiological role of oxidants in cellular proliferation and host defence. Similarly, increase in ROS may also be detrimental and lead to cell death or to acceleration in ageing and age-related diseases. Traditionally, the impairment caused by increased ROS is thought to result from random damage to proteins, lipids and DNA. In addition to these effects, a rise in ROS levels may also constitute a stress signal that activates specific redox sensitive signalling pathways. Once activated, these diverse signalling pathways may have either damaging or potentially protective functions [11].

In, India Ayurvedic medicine has great importance to treat the diabetes and its complications. Since ancient period, it gains more popularity due to its less toxic effects and more efficacious. Many herbs have been shown to have antidiabetic action in both human and animals [12].

The ethnobotanical information reports about 800 plants that may possess antidiabetic potential [13], Folk medicine for diabetes from Rayalaseema reports 26 plants with antidiabetic activity, one such plant is *Boswellia sarrata,* which showed antidiabetic effect in diabetic rats [14,15], other species of this genus *Boswellia ovalifoliolata* has wide range of medicinal uses [16], along with antidiabetic activity [17]. *Boswellia ovalifoliolata* Bal and Henry, a member of Burseraceae, is an endemic species [18], which occurs at an altitudinal range of 250 - 600 m on Seshachalam hill ranges of Palakonda region of Eastern Ghats of India. This plant is vernacularly known as Konda guggilum, Adavi sambrani. The plant is used by tribals to treat number of medicinal ailments. The plant is over exploited for its medicinal uses; especially the leaf juice is used to prevent throat ulcers [19], the gum is used to cure amoebic dysentery and hydroceal [20]. Stem bark is used to cure rheumatic pains [21]. Equal mixture of Gum and stem bark one tea spoon full is given daily with sour milk on empty stomach for a month to cure stomach ulcers [22]. It is used in synthesizing silver nanoparticles, which can provide a new platform to this plant making it's a value added tree for nanotechnology based medicine in future [23]. But there are no significant reports on antioxidant activity of the stem bark of *Boswellia ovalifoliolata,* hence this study was taken up for the evaluation of antioxidant effect of SBBO in STZ induced diabetic rats.

Materials and Methods

Collection of Plant material

The stems bark of *Boswellia ovalifoliolata* (SBBO) Bal and Henry was collected from Tirumala hills, Tirupati, Andhra Pradesh, India. It was identified and authenticated by the Taxonomist of the Herbarium, Department of Botany, Sri Venkateswara University, Tirupati, Andhra Pradesh, India. A voucher specimen (Herbarium Accession Number: 516) has been kept in our library herbarium for future reference. The stem bark of *Boswellia ovalifoliolata* was dried in shade, powdered and the powder was used for the preparation of different solvent extracts.

Preparation of Aqueous extract

To prepare aqueous extract the SBBO powder (1 kg) was soaked in distilled water (3 volumes) in a glass jar for 2 days at room temperature and the solvent was filtered. This was repeated 3 to 4 times until the filtrate gave no coloration. The filtrate was concentrated under reduced pressure in the Buchi rotavapour R-200 and finally freeze dried. The yield of the extract was 26% w/w.

Phytochemical analysis of the aqueous extract

Aqueous extract of SBBO was screened for the presence of various phytochemical constituents using standard methods of phytochemical analysis [24].

Induction of diabetes

Diabetes was induced in male Wistar albino rats aged 2-3 months (180-200 g body weight) by intraperitoneal administration of STZ (single dose of 50 mg/kg b.w.) dissolved in freshly prepared 0.01M citrate buffer, pH 4.5. After 72 h rats with marked hyperglycemia (FBG 250 mg/dI) were selected and used for the study [25]. All the animals were allowed free access to tap water and pellet diet and maintained at room temperature in plastic cages, as per the guidelines of Institute Animals Ethics committee. This study was approved by institutional animal ethics committee vide Resolution no: 31/2012-2013/(i)/a/CPCSEA/ IAEC/SVU/ CAR-YKP dt. 01-07-2012.

Experimental design

The rats were divided into 5 groups and each group consisted of 6 rats as given below.

Group 1. Normal untreated rats

Group 2. Normal rats treated with 200mg of AESBBO / kg bw/ day/ for 40 days

Group 3. Diabetic untreated rats

Group 4. Diabetic rats treated with 200mg of AESBBO / kg bw/ day/ for 40 days

Group 5. Diabetic rats treated with 20mg glibenclamide / kg bw/ day / for 40 days

AESBBO or glibenclamide was administered to the rats every day morning for 40 days by gastric intubation using oral gavage. All the five groups of rats were sacrificed on the 41th day after an overnight fast, by anesthetizing with anaesthetic ether and further by cervical dislocation. Different tissues including liver and kidney were collected and immediately frozen until the use, for measurement of lipid peroxides and activities of antioxidant enzymes.

Analytical procedures

The levels of TBARS in tissues were estimated by the method of Fraga *et al.,* 1988[26]. CAT activity was assayed following the method of Sinha., 1972 [27]. SOD activity was assessed according to the method of Kakkar *et al.,* 1984 [28]. GPx activity was measured as described by Rotruck *et al.,* 1973 [29]. GST activity was estimated according to the method of Habig *et al.* 1974[30].

Statistical analysis

The results were expressed as mean ± S.D. The statistical analysis of results was carried out using one-way analysis (ANOVA) followed by DMRT.

Results

Phytochemical analysis of Aqueous extract of SBBO

Phytochemical analysis of the aqueous extract of stem bark of *Boswellia ovalifoliolata* has shown the presence of Flavonoids, Saponins, Carbohydrates and Tannins.

Table.1 and table.2 Show the effect of long term treatment with the AESBBO on lipid peroxides, activities of CAT, SOD, GPX and GST.

Table.1 and Table.2 show the liver and kidney (respectively) levels of TBARS, activities of SOD, CAT, GPx and GST in the normal and experimental groups of rats. There was a significant increase in the levels of TBARS, CAT activity and a significant decrease in the activities of SOD, GPx and GST in both tissues of diabetic rats compared to those in normals. The treatment with AESBBO decreased the levels of TBARS, CAT activity and significantly increased activities of SOD, GPx and GST in liver and kidney of diabetic rats. Treatment of the 5th group of rats with glibenclamide resulted in similar changes in the levels of lipid peroxides and antioxidant enzyme activities.

Discussion

The use of plant products in the treatment of diabetes mellitus is becoming advantageous due to the presence of several bioactive compounds with therapeutic potential. In recent years, several researchers have studied the worth of different medicinal plants in controlling DM and delaying the long term effects of DM. *Boswellia ovalifoliolata* is one of the herbs mentioned in all ancient scriptures of Ayurved. The present study was conducted to evaluate the beneficial effects of Aqueous extract of stem bark of *Boswellia ovalifoliolata* (AESBBO) on lipid peroxidation and antioxidant status in STZ induced diabetic rats. It has been stated that STZ diabetic animals may exhibit most of the diabetic complications mediated through oxidative stress (Ozturia *et al.,* 1996) [31]. Oxidative stress depicts the existence of products called free radicals and reactive oxygen species (ROS) which are formed under normal physiological conditions but become deleterious when not being quenched by the antioxidant systems [32]. There are convincing experimental and clinical evidences that the generation of reactive oxygen species is increased in both types of diabetes and that the onset of diabetes is closely associated with oxidative stress [33,34]. Free radicals are formed disproportionately in diabetes by glucose auto oxidation, polyol pathway and non-enzymatic glycation of proteins [35].

STZ - induced hyperglycemia induces free radical generation which there by leads to DNA damage, protein degradation, lipid peroxidation and finally culminating into damage to various organs of the body like liver, kidney, brain, eyes, enzymes and development of complications of *diabetes mellitus* [36]. Implication of oxidative stress in the pathogenesis of diabetes mellitus is suggested not only by oxygen free radical generation but also due to non-enzymatic protein glycosylation, auto-oxidation of glucose, impaired antioxidant enzyme, and formation of peroxides [37,38]. Increased oxidative stress as measured by the index of lipid peroxidation has been shown to be increased in both insulin-dependent (IDDM), and non-insulindependent diabetes mellitus (NIDDM) [39] and it could cause initial ²-cell damage in type I diabetes or impaired insulin production, release, or function in type II diabetes [40,41]. The increased lipid peroxidation in the diabetic animals may be due to the observed remarkable increase in the concentration of TBARS and hydroperoxides (lipid peroxidative markers) in the liver and kidney of diabetic rats (Stanely *et al,.* 2001) [42].

In our study STZ was used to induce DM in rats rather than alloxan. At low dose, STZ (50 mg/kg bw) partially destructs the ²-cells, which secreted insufficient insulin causing type 2 diabetes [43]. It is widely accepted animal model and reported to resemble human hyperglycaemic non ketotic diabetes mellitus [44], is often associated with kidney hypertrophy which may contribute to end stage renal damage, hepatotoxicity, oxidative stress and hypercholesterolemia [45,46].

Hypoinsulinaemia in diabetes increases the activity of the enzyme fatty acyl coenzyme A oxidase, which intiates ²-oxidation of fatty acids, resulting in lipid peroxidation [47]. Increased lipid peroxidation impairs membrane function by decreasing membrane fluidity and changing the activity of membrane-bound enzymes and receptors. The products of lipid peroxidation are harmful to most cells in the body and are associated with a variety of diseases, such as atherosclerosis and brain damage [48].

In the present study, we observed a significant increase in lipid peroxide levels (TBARS) in the liver and kidney of diabetic rats compared to normal rats. Administration of AESBBO or glibenclamide decreased the levels of TBARS in the liver and kidney of diabetic rats. This shows that AESBBO might protect the tissues (liver and kidney) against the cytotoxic action and oxidative stress of streptozotocin.

DM is associated with increased formation of free radicals and decrease in antioxidant potential. Due to these events, the balance normally present in cells between radical formation and the protection against them is disturbed [49]. An imbalance of the oxidant / antioxidant defence systems results in alterations in the activity of antioxidant enzymes, such as SOD, CAT,GR, GPx, and impaired glutathione metabolism [50]. The present data indicates that STZ-induced diabetes disrupts actions of liver and kidney antioxidant enzymes. The decreased activities of these result in accumulation of superoxide (O_2), hydrogen peroxide (H_2O_2), and hydroxyl radical (OH) that reduce the activity of these enzymes [51,52].

In our study, the activities of SOD, GPx and GST were decreased in diabetic rats compared to normal rats, which could be due to free radical-induced inactivation and glycation of the enzymes in diabetic state [53]. On long-term treatment of diabetic rats AESBBO had reversed the activities of these enzymatic antioxidants, This means that the extracts can reduce the potential glycation of enzymes or they may reduce the production of reactive oxygen free radicals and improve the activities of antioxidant enzymes.

In our study the activity of CAT was significantly increased in liver and kidney of diabetic untreated rats. The possible explanation for the increases in catalase activity is that it could be a compensatory mechanism to prevent tissue damage by the increased levels of H_2O_2 and decreased levels of GPx,. In diabetes,it is thought that hypoinsulinemia increases the activity of the enzyme, fatty acyl coenzyme A oxidase, which initiates ± oxidation of fatty acids, resulting in increased levels of H_2O_2. The CAT activity was restored

Table - 1

Effect of long term treatment with the AESBBO on TBARS levels and antioxidant enzyme activities in the livers of different experimental animals

Group	Lipid Peroxides (nmoles MDA/ml)	Catalase (U/mg Protein)	Glutathione Peroxidase (U/ mg protein)	Superoxide Dismutase (U/ mg protein)	Glutathione-S-Transferase (U/ mg protein)
1	0.123±0.001 [a]	19.1±0.52 [a]	0.257±0.018 [e]	16.0±0.68 [c]	21.4±0.33 [d]
2	0.130±0.001 [b]	20.4±0.25 [b]	0.232±0.017 [d]	17.4±0.43 [d]	24.0±0.40 [e]
3	0.232±0.007 [e]	46.4±0.30 [e]	0.072±0.014 [a]	6.4±0.33 [a]	8.7±0.45 [a]
4	0.157±0.005 [d]	21.8±0.59 [c]	0.165±0.012 [c]	13.1±0.59 [b]	18.3±0.27 [c]
5	0.146±0.004 [c]	23.3±0.28 [d]	0.128±0.011 [b]	12.0±0.55 [b]	16.0±0.55 [b]
F value	500.906	452.1	150.843	362.044	119.478
Significance	0	0	0	0	0

Values are given as mean ± S.D from six rats in each group.
Values not sharing a common superscript letter differ significantly at p< 0.01 (DMRT).

Table – 2

Effect of long term treatment with AESBBO on TBARS levels and antioxidant enzyme activities in the Kidneys of different experimental animals.

Group	Lipid Peroxides (nmoles MDA/ml)	Catalase (U/mg Protein)	Glutathione Peroxidase (U/ mg protein)	Superoxide Dismutase (U/ mg protein)	Glutathione-S-Transferase (U/ mg protein)
1	0.134±0.003 [a]	34.4±0.35 [b]	0.229±0.008 [d]	34.03±0.73 [d]	26.58±0.42 [d]
2	0.135±0.002 [a]	32.1±0.74 [a]	0.235±0.012 [d]	35.95±0.76 [e]	25.06±0.69 [c]
3	0.253±0.002 [d]	60.3±0.30 [e]	0.128±0.007 [a]	14.98±0.65 [a]	9.56±0.48 [a]
4	0.163±0.002 [b]	38.5±0.40 [c]	0.198±0.039 [c]	31.05±0.61 [c]	22.86±0.69 [b]
5	0.174±0.003 [c]	40.3±0.33 [d]	0.171±0.012 [b]	26.88±0.45 [b]	28.33±0.68 [e]
F value	179.95	356.9	28.64	97.55	90.97
Significance	0	0	0	0	0

Values are given as mean ± S.D from six rats in each group.
Values not sharing a common superscript letter differ significantly at p< 0.01 (DMRT).

to near normal in diabetic rats treated with AESBBO, which might be due to decreased LPO levels and increased insulin secretion.

Various studies in the past reported conflicting results regarding the status of antioxidant enzymes in diabetes [54,55]. Majority of authors reported the decreased enzymatic antioxidant activites (SOD,CAT,GPx and GST) in tissues of diabetic rats. [56,57].

In conclusion the present study showed that the *Boswellia ovalifoliolata* has significant antioxidant activity. The bioactive component(s) responsible for the observed activites are not precisely known but it may be one or more of the phytochemical constituents present in the aqueous extract of stem bark of *Boswellia ovalifoliolata*.

References

[1] Anitha B, Mohan VR, Athiperumalsami T, Suthaa S. Ethnomedicinal Plants Used by the Kanikkars of Tirunelveli District, Tamil Nadu, Ethnobotanical Leaflets 2008; 12: 171-180.

[2] Kumar S, Kumar V, Prakash O. Antihyperglycemic, antihyperlipidemic potential and histopathological analysis of ethyl acetate fraction of Callistemon lanceolatus leaves extract on alloxan induced diabetic rats. J Exper Integr Med 2011; 1(3):185-190.

[3] Oyedemi SO, Adewusi EA, Aiyegoro OA, Akinpelu DA. Antidiabetic and haematological effect of aqueous extract of stem bark of Afzelia africana (Smith) on streptozotocin-induced diabetic Wistar rats. Asian Pac J Trop Dis 2011; 1(5): 353-358.

[4] Chehade JM, Mooradian AD. A Rational Approach to Drug Therapy of Type 2 Diabetes Mellitus, Disease Management. Drugs 2000; 60 (1): 95-113.

[5] Kumar S, Kumar V, Prakash O. Antidiabetic and antihyperlipidemic effects of Dillenia indica (L.) leaves Extract. Braz J Pharm Sci 2011; 47 (2): 1-6.

[6] Kumar S, Kumar V, Prakash O. Antidiabetic, hypolipidemic and histopathological analysis of Dillenia indica (L.) leaves extract on alloxan induced diabetic rats. Asian Pac J Trop Med 2011; 4 (5): 347-352.

[7] Girija K, Lakshman K, Udaya Chandrika, Sabhya Sachi Ghosh, Divya T. Anti-diabetic and anti-cholesterolemic activity of methanol extracts of three species of Amaranthus. Asian Pac J Trop Biomed 2011; 1(2):133-138.

[8] Thirumalai T, Viviyan Therasa S, Elumalai EK, David E. Hypoglycemic effect of Brassica juncea (seeds) on streptozotocin induced diabetic male albino rat. Asian Pac J Trop Biomed 2011; 1 (4): 323-325.

[9] Moussa SA. Oxidative stress in Diabetes Mellitus. Romanian J Biophys 2008;18(3):225-236.

[10] Boguslaw Lipinski. Pathophysilogy of Oxidative stress in Diabetes mellitus. Journal of Diabetes and its Complications 2001; 15: 203-210.

[11] Toren Finkel & Nikki J Holbrook. Oxidative stress and the biology of ageing. Nature 2000;408: 239-247.

[12] Bhattaram VA, Ceraefe M, Kohlest C, Vest M, Deundorf H. Pharmacokinetics and Bioavailability of herbal medicinal products. Phytomed 2002; 9: 1-36.

[13] Alarcon Aguilara FJ, Roman Ramos R, Perez Gutierrez S, A Aguilar Contreras CC. Contreras Weber JL. Flores Saenz, Study of the antihyperglycaemic effect of plants used as antidiabetics, Journal of Ethanopharmacology 61 (1998) 101–110.

[14] Ahmadpour F, Namjoyan F, Azemi M, Khodayar M, Darvish Padok A, Panahi M, Antioxidant capacity and anti-diabetic effect of Boswellia serrataa aqueous extract in female diabetic rats, Research in Pharmaceutical Sciences 5 (2012) 7.

[15] Mohamad Ebrahim Azemi, Foroogh Namjoyan, Mohammad Javad Khodayar, Forouzan Ahmadpour, Azam Darvish Padok, Marziyeh Panahi, Jundishapur Journal of Natural Pharmaceutical Products (2012).

[16] Savithramma N, Studies of Boswellia ovalifoliolata BAL. AND HENRY- AN, Endemic and endangered medicinal plant, The Bioscan 5 (3) (2010) 359-362.

[17] Madhavachetty K, Sivaji K, Tulasirao K, Flowering plants of chittoor district, Andhra Pradesh, India, Third edition, published by student offset printers, Tirupati, (2011) 61.

[18] Ahmedullah and Nayar MP, Endemic plants of the Indian region, Botanical Survey of India, Howrah, 1 (1986) 38-39.

[19] Savithramma N, Sulochana CH, Endamic medicinal plants from Tirumala hills, A.P, India, Fitoterapia (1998) 253-254.

[20] Sudhakar, Pharmacognosy of some indigenous medicinal plants of Chittoor district, Andhrapradesh, India, Fitoterapia. (1998) 390-400.

[21] Latheef SA, Prasad B, Bavaji M, Subramanyam G, A database on endemic plants at Tirumala Hills in India, Bioinformation 2 (2008) 260–262.

[22] Nagaraju N, KN Rao, A survey of plant crude drugs of Rayalaseema, Journal of Ethanopharmacology 29 (1990) 137-158.

[23] Ankanna S, Prasada TNVKV, Elumalaib EK, Savithramma N, production of biogenic Silvernanoparticles using boswellia ovalifoliolata stem bark, Digest Journal of Nanomaterials and Biostructure 5 (2) (2010) 369 –372.

[24] Harborne JB, Phytochemical methods, analysis, A guide to modern techniques of plant, 3rd ed. Spinger international, India (2005).

[25] Gupta S, Kataria M, Gupta PK, Murganandan S, Yashroy RC. Protective role of extract of Neem seeds in diabetes caused by Streptozotocin in rats, J. Ethnopharmcol 2004; 90: 185-189.

[26] Fraga CG, Leibovitz BE, Toppel AL. Lipid peroxidation measured as TBARS in Tissue Slices. Characterisation and comparison with homogenatye and microsome. Free RadBio Med 1988; 4, 155-161.

[27] Sinha KA. Colarimetric assay of Catalase. Anal Biochem 1972:47, 389-394.

[28] Kakkar P, Das D, Viswanathan PNA modified spectrophotometric assay of Superoxide Dismutase. Ind J Biochem Biophys 1984; 21, 130-132.

[29] Rotruck JT, Pope AL, Ganther HE, Swanson AB. Selenium: Biochemical role as A component of Glutathione peroxidise. Science 1973; 179, 588-590.

[30] Habig WH, Pabst MJ, Jakoby WB. Glutathione transferase. The first enzymatic step in mercapturic acid foramation. J Biol Chem 1974; 249,7130-7139.

[31] Ozturia Y, Altan VM, Yildizoglu A, 1996. Effect of experimental diabetes and insulin on smooth muscle functions. Pharma Review, 48: 69.

[32] Fang YZ, Yang S, Wu G, Free radical, antioxidant and nutrition, Nutrition, 2002, 18, 872–890.

[33] Johansen JS, Harris, AK, Rychly DJ, Ergul A, Oxidative stress and the use of antioxidants in diabetes: Linking basic science to clinical practice, Cardiovascular Diabetology, 2005, 4, 5–9.

[34] Rosen P, Nawroth PP, King G, Moller G, Tritschrev HJ, Packer L, The role of oxidative stress in the onset and progression of diabetes and its complication, Diabetesmetabolism Research and Reviews, 2001, 17, 189–212.

[35] Obrosova IG, Vanlteysen C, Fathallah L, Cao X, Greene DA, Stevens MJ., An aldose reductase inhibitor reverses early diabetes-induced changes inperipheral nerve function, FASEB J., 2002, 16, 123–125.

[36] Maritim, A.C., R.A. Sanders, J.B. Watkins, Diabetes, oxidative stress and antioxidants: a review, Journal of Biochemical and Molecular Toxicology, 2003, 17, 24–38.

[37] Vincent AM, Russell JW, Low P, Feldman EL. Oxidative stress in the pathogenesis of Diabetic neuropathy. Endocr Rev 2004; 25, 621-28.

[38] Pari L, Latha M. Antidiabetic effect of Scoparia dulcis: effect on lipid peroxidation in streptozotocin diabetes. Gen Physio Biophys 2005; 24,13-26.

[39] Atalay M, Laaksonen DE, Diabetes, oxidative stress and physical exercise, J.Sports Sci. Med., 2002, 1, 1–4.

[40] Bonnefont Rousselot D, Bastard JP, Jaudon MC, Delattre J, Consequences of the diabetic status on the oxidant/ antioxidant balance, Diabetes Metab., 2000, 26, 163–176.

[41] West IC, Radicals and oxidative stress in diabetes. Diabetic Med., 2000, 17, 171–180.

[42] Stanely MP, Menon VP, 2001. Antioxidant action of *Tinospora cardifolia* root extract in alloxan diabetic rats. Phytotherapy Research, 15: 213.

[43] Gomes A, Vedasiromoni JR, Das M, Sharma RM, Ganguly DK. Antihyperglycemic effect of black tea (Camellia sinensis) in rat . J. Ethnopharmacol. 2001; 27, 243-275.

[44] Weir GC, Clore ET, Zmachiroski CJ, Bonner-Weri S Islet secrection in a new experiment Model for non-insulin depent diabetes. Diabetes 1981 5: 30-590.

[45] Heidland A, Ling H, Vamvakas S, Paczek L (1996) Imparied proteolytic activity as a Potential cause of progressive renal disease. Miner Electrolyte Metab 22: 157-61.

[46] Rabkin R, Schechter P, Shi JD, Boner G Protein turnover in the hypertrophy in kidney. Miner Electrolyte Metab 1966; 22: 153-6.

[47] Horie S, Ishii H, Suga T, Changes in peroxisomal fatty acid oxidation in diabetic rat liver, J. Biochem., 1981, 90, 1691–1696.

[48] Acworth IN, Mccabe DR, Maher T, The analysis of free radicals, their reaction products and antioxidants, in: S.I. Baskin, H. Salem (Eds.), Oxidants, Antioxidants and free radicals, Taylor and Francis, Washington, DC, 1997, Chapter 2.

[49] Nazirogilu M, Butterworth P. Protective effects of moderate exercise with dietary Vitamin C and E on blood antioxidative defense mechanism in rats with streptozotocin induced diabetes. Can. J. Appl. Physiol. 2005; 30, 172-185.

[50] Maritim AC. Sanders RA., Watkins JB. Diabetes, oxidative stress and antioxidants. A review. J. Biochem. Mol. Toxicol. 2003; 17, 24-38.

[51] Kaleem M, Asif M, Ahmed QU, Bano B. Antidiabetic and antioxidant activity of Annona squamosa exctract in streptozotiocin-induced diabetic rats. Singapore Med. 2006; J. 47,670-675.

[52] Vincent AM, Russell JW, Low P, Feldman EL. Oxidative stress in the pathogenesis of diabetic neuropathy. Endocr Rev 2004; 25, 612-28.

[53] AI-Azzawie HF, Alhamdani MS. Hypoglycaemic and antioxidant effect of oleuropein In alloxan-induced diabetic rabbits. Life Sci. 2006; 78 (12), 1371-1377.

[54] Kaji H, Jurasaki M, Ito K. Increased lipid peroxide value and glutathione peroxide activity In blood plasma of type 11 diabeteic women. Klin Wochnschr 1985; 63, 765-8.

[55] Matkovics B, Kotorman M, Varga IS, Hai DW, Varga C. Proantioxidant and rheological studied in the blood of type 2 diabetic patients. Acta physiol. Hung. 1997, 85,197-112.

[56] Goplasamy Rajiv Gandhi, Savarimuthu Ignacimuthu, Michael Gabriel Paulraj. Solanum Torvum Swartz. Fruit containing phenolic compounds shows antidiabetic and antioxidant Effectsin streptozotocin induced diabetic rats. Food and Chemical Toxicology 49; 2725-2733.

[57] Selvan VT, Manikandan L, Senthil kumar GP, Suresh R, Kakoti BB, Gomathi P, Kumar DA, Saha P, Gupta M, Mazumder UK. Antidiabetic and Antioxidant Effect of Methanol Exctract of Artanema sesamoides in Streptatozocin-Induced Diabetic Rats International Journal of Applied Research in Natural Products Vol. 1 (1), 2008; pp. 25-33.

ISSN 0973 – 9874

Synthesis of Polyhydroquinoline *via* the Hantzsch Reaction using PASS as Heterogeneous Catalyst under Solvent Free-conditions

C. RADHAKRISHNA, V. SRINIVASA RAO AND T. PARTHASARATHY*

Department of Chemistry, Osmania University, Hyderabad-500 007. INDIA
Tel : +91 9949652118; +91-40-23393530, fax: +91-40-23306136

An alternative polyhydroquinoline derivatives *via* the Hantzsch reaction using a variety of aldehydes, 5,5-dimethyl-1,3-cyclohexanedione or 1,3-cyclohexanedione, ²-keto compounds, and ammonium acetate in the presence of Poly Aniline Sulphate Salt (PASS) as a heterogeneous catalyst in a solvent-free media at 90 °C. The present methodology offers several advantages such as excellent yields, simple procedure, short reaction times (10-25 min.) and milder conditions and the catalyst exhibited remarkable reusable activity.

Key words: PASS, 5,5-dimethyl-1,3-cyclohexanedione, 1,3-cyclohexanedione, 2,4-pentadione, alkyl acetoacetate, solvent-free media, reusable activity.

Introduction

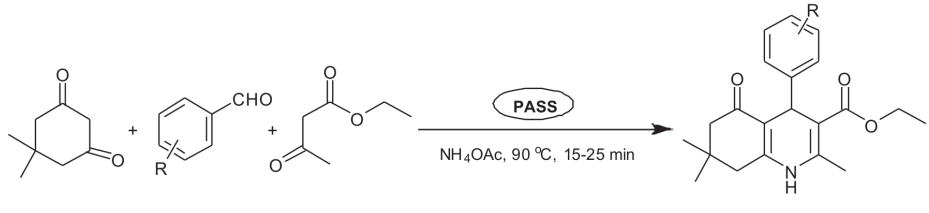
Multicomponent reactions (MCRs) are of increasing importance in organic and medicinal chemistry. One such reaction that belongs to this class is venerable Hantzsch synthesis for dihydropyridines (DHPs). In 1882, Hantzsch[1] first reported synthesis of dihydropyridine by condensation of an alddehyde, ²-keto ester and ammonia. Till date the chemistry of DHPs and their cyclic analogs polyhydroquinolines had received special attention and reviewed in several surveys of literature[2,3]. 1, 4-DHPs have attracted organic chemists among the different isomers of dihydropyridines and interest in these compounds is increasing progressively. Investigations along these lines include the synthesis of model compounds NADPH-analogues of 1,4-dihydronicotinamide and their involvement in hydrogen transfer reactions. A variety of catalysts have been reported for the synthesis of polyhydroquinoline derivatives such as isopropanol[4], solid support[5], MW[6], ionic liquid[7], TBAHS[8], $Yb(OTf)_3$[9] and iodine[10]. The search for new reagents capable of mediating these reactions is still a matter of much concern.

In view of current interest in catalytic processes, there is a need to develop a catalytic method for the synthesis of polyhydroquinoline derivatives. This method should allow for increase of selectivity, maximize the use of starting materials and facilitate the easy separation of the final reaction mixture including the efficient recovery and reusability of the catalyst.

In continuation of our studies directed towards the development of practical, and environmentally friendly procedures for some important organic reactions, we have developed the applicability of a novel Poly-Aniline Sulfate salt [PASS] (10 mol%) for efficient, convenient and facile synthesis of polyhydroquinoline derivatives by one-pot reaction of aryl aldehydes, 5,5-dimethyl-1,3-cyclohexanedione, ethyl acetoacetate and ammonium acetate in solvent-free media at 90 °C (**Scheme 1**).

Experimental

All the commercial reagents and solvents were used without further purification unless otherwise stated. Melting points



1 2a-o 3 4a-o

Scheme 1

*Address for correspondence: email : sarathychem@gmail.com

were recorded on a Buchi 535 melting point apparatus and are uncorrected. All the reactions were monitored by thin layer chromatography performed on precoated silica gel $60F_{254}$ plates (Merck). Compounds were visualized with UV light at 254 nm and 365 nm, I_2 and heating plates after dipping in 2% phosphomolybdic acid in 15% aq. H_2SO_4 soln. IR spectra were recorded on a Perkin-Elmer 683 or a 1310 FT-IR spectrometers with KBr pellets. NMR spectra were recorded on a Varian Unity-400 MHz and BRUKER AMX 300 spectrometers using TMS as an internal standard. Mass spectra were recorded on a VG Micromass 7070H and a Finnigan Mat 1020B mass spectrometers operating at 70eV.

General Procedure **for Synthesis of Polyhydroquinolines (4a-o):** A mixture of benzaldehyde (**2a**, 0.21 g, 2.0 mmol), 5,5-dimethyl-1,3-cyclohexanedione (**1**, 0.28 g, 2.0 mmol), ethyl acetoacetate (**3**, 0.26 g, 2.0 mmol), ammonium acetate (0.23 g, 3.0 mmol) and PASS (10 mol%) was heated at 90 °C for 15 min. After completion of reaction as indicated by TLC, the resulting solid product was diluted with methanol, filtered (to remove the catalyst) and filtrate was concentrated in *vaccuo* to a solid residue which was purified by recrystallization in ethanol (3 mL) to obtain title compound.

2,7,7'-Trimethyl-5-oxo-4-pheny-1,4,5,6,7,8-hexahydroquinoline-3-carboxylic acid ethyl ester (4a): Yield: 90%) as yellow crystalline solid., m.p. 230-232 °C. IR (KBr): υ 3288, 1698 cm^{-1}. ^1H NMR (DMSO-d_6): 0.86 (s, 3H, CH_3),1.03 (s, 3H, CH_3), 1.15 (t, 3H, J = 7.2 Hz, CH_3), 2.04 (dd, 2H, CH), 2.27 (s, 5H, CH_3 and CH_2), 3.96 (q, 2H, J = 7.2 Hz, -0CH_2-), 4.85 (s, 1H, CH), 7.11 (m, 5H, ArH), 8.55 (brs, 1H, NH). ESI-MS: m/z 340 $[M+H]^+$.

2,7,7'-Trimethyl-5-oxo-4-(4-methoxyphenyl)-1,4,5,6,7,8-hexahydroquinoline-3-carboxylic acid ethyl ester (4b): Yield: 93% as colorless solid., m.p. 259-260 °C. IR (KBr): υ 3276, 1701 cm^{-1}. ^1H NMR (DMSO-d_6): δ 0.89 (s, 3H, CH_3), 1.02 (s, 3H, CH_3), 1.18 (t, 3H, J = 7.3 Hz, CH_3), 2.02 (dd, 2H, -CH_2-), 2.28 (s, 5H, CH_3 and CH_2), 3.69 (s, 3H, Ar-OCH_3), 3.88 (q, 2H, J = 7.3 Hz, -OCH_2-), 4.77 (s, 1H, CH), 6.64 (d, 2H, J = 9.3Hz, ArH), 7.06 (d, 2H, J = 9.3 Hz, ArH), 8.69 (brs,1H, NH). ESI-MS: m/z 370 $[M+H]^+$.

2,7,7'-Trimethyl-5-oxo-4-(4-chlorophenyl)-1,4,5,6,7,8-hexahydroquinoline-3-carboxylicacid ethyl ester (4c): Yield: 93% as colorless solid., m.p. 230-232 °C. IR (KBr): υ 3274, 1706 cm^{-1}. ^1H NMR (DMSO-d_6): δ 0.90 (s, 3H, CH_3), 1.06 (s, 3H, CH_3), 1.20 (t, 3H, J = 7.5 Hz, CH_3), 2.07 (dd, 2H, CH-$_2$), 2.30 (s, 5H, CH_3 and CH_2), 4.00 (q, 2H, J = 7.5 Hz, -OCH_2-), 4.85 (s, 1H, CH), 7.10, 7.18 (2d , 4H, J = 8.0 Hz, ArH), 8.70 (brs, 1H, NH). ESI-MS: m/z 374 $[M+H]^+$.

2,7,7'-Trimethyl-5-oxo-4-(4-nitrophenyl)-1,4,5,6,7,8-hexahydroquinoline-3-carboxylic acid ethyl ester (4d): Yield: 86% as yellow colored solid., m.p. 242-243 °C. IR (KBr): υ 3506, 1619 cm^{-1}. ^1H NMR ($CDCl_3$+DMSO-d_6): δ 0.90 (s, 3H, CH_3), 1.08 (s, 3H, CH_3), 1.19 (t, 3H, J = 7.3 Hz, CH_3), 2.10 (dd, 2H, -CH_2-), 2.36 (s, 5H, CH_3 and CH_2), 4.00 (q, 2H, J = 6.5 Hz, -OCH_2-), 5.03 (s,1H, CH), 7.42 (d, 2H, J = 7.4 Hz, ArH), 8.03 (d, 2H, J = 7.4 Hz, ArH), 8.65 (brs, 1H, NH). ESI-MS: m/z 385 $[M+H]^+$.

2,7,7'-Trimethyl-5-oxo-4-(4-methylphenyl)-1,4,5,6,7,8-hexahydroquinoline-3-carboxylicacid ethyl ester (4e): Yield: 94% as colorless solid., m.p. 266-267 °C. IR (KBr): υ 3275, 1701 cm^{-1}. ^1H NMR ($CDCl_3$+DMSO-d_6): δ 0.91 (s, 3H, CH_3), 1.06 (s, 3H, CH_3), 1.20 (t, 3H, J = 6.5 Hz, CH_3), 2.10 (dd, 2H, -CH_2-), 2.32 (s, 3H, ArCH_3), 2.32 (s, 5H, CH_3 and CH_2), 4.00 (q, 2H, J = 6.5 Hz, -OCH_2-), 4.87 (s, 1H, CH), 6.92 (d, 2H, J = 8.0 Hz, ArH), 7.10 (d, 2H, J = 8.0 Hz, ArH), 8.28 (brs, 1H, NH). ESI-MS: m/z 354 $[M+H]^+$.

2,7,7'-Trimethyl-5-oxo-4-(2-chlorophenyl)-1,4,5,6,7,8-hexahydroquinoline-3-carboxylic acid ethyl ester (4f): Yield; 90% as crystalline solid., m.p. 211-212 °C. IR (KBr): υ 3291, 1697 cm^{-1}. ^1H NMR ($CDCl_3$+DMSO-d_6): δ 0.91 (s, 3H, CH_3), 1.06 (s, 3H, CH_3), 1.15 (t, 3H, J = 7.2 Hz, CH_3), 2.07 (dd, 2H, -CH_2-), 2.28 (s, 5H, CH_3 and CH_2), 3.98 (q, 2H, J = 2.1 Hz, -OCH_2-), 5.22 (s, 1H, CH), 7.00 (t, 1H, J = 7.2 Hz, ArH), 7.18 (t, 1H, J = 7.2 Hz, ArH), 7.15 (d, 1H, J = 7.9 Hz, ArH), 7.30 (d, 1H, J = 7.9 Hz, Ar-H), 8.62 (brs, 1H, NH). ESI-MS: m/z: 374 $[M+H]^+$.

2,7,7'-Trimethyl-5-oxo-4-(4-hydroxy-5-methoxyphenyl)-1,4,5,6,7,8-hydroquinoline-3-carboxylic acid ethyl ester (4g): Yield: 92% as cream colored solid., m.p. 236-237 °C. IR (KBr): υ 3397, 1697 cm^{-1}. ^1H NMR ($CDCl_3$+DMSO-d_6): δ 0.90 (s, 3H, CH_3), 1.05 (s, 3H, CH_3), 1.20 (t, 3H, J = 7.0 Hz, CH_3), 2.04 (dd, 2H, -CH_2-), 2.25 (s, 5H, CH_3 and CH_2), 3.72 (s, 3H, OCH_3), 4.00 (q, 2H, J = 7.0 Hz, -OCH_2-), 4.73 (s, 1H, CH), 6.52 (s, 2H, ArH), 6.72 (s,1H, ArH), 8.00 (s,1H, OH), 8.60 (brs, 1H, NH). ESI-MS: m/z 386 $[M+H]^+$.

2,7,7'-Trimethyl-5-oxo-4-(3-methoxyphenyl)-1,4,5,6,7,8-hexahydroquinoline-3-carboxylic acid ethyl ester (4h): Yield: 91% as colorless solid, m.p. 202-204 °C. IR (KBr): υ 3300, 1696 cm^{-1}. ^1H NMR ($CDCl_3$+DMSO-d_6): δ 0.87 (s, 3H, CH_3), 1.00 (s, 3H, CH_3), 1.15 (t, 3H, J = 7.6 Hz, CH_3), 2.02 (dd, 2H, -CH_2-), 2.38 (s, 5H, CH_3 and CH_2), 3.78 (s, 3H, OCH_3), 3.98 (q, 2H, J = 7.6 Hz, -OCH_2-), 4.81 (s, 1H, CH), 6.50 (d, 1H, J = 5.3 Hz, ArH), 6.70 (s, 1H, ArH), 6.72 (d, 1H, J = 7.6 Hz, ArH) 6.98 (t, 1H, J = 7.6 Hz, ArH), 8.35 (brs, 1H, NH). ESI-MS: m/z 370 $[M+H]^+$.

2,7,7'-Trimethyl-5-oxo-4-(2-nitrophenyl)-1,4,5,6,7,8-hexahydroquinoline-3-carboxylic acid ethyl ester (4i): Yield: 87% as yellow colored solid, m.p. 210-212 °C. IR (KBr): υ 3292, 1698 cm^{-1}. ^1H NMR ($CDCl_3$+DMSO-d_6): δ 0.88 (s, 3H, CH_3), 1.05 (s, 3H, CH_3), 1.11 (t, 3H, J = 7.3 Hz, CH_3), 2.05 (dd, 2H, -CH_2-), 2.34 (s, 5H, CH_3 and CH_2), 4.00 (q, 2H, J = 7.3 Hz, -OCH_2-), 5.72 (s, 1H, CH), 7.20 (t, 2H, J = 6.5 Hz, ArH), 7.42 (m, 2H, ArH), 7.65 (d,

1H, *J* = 8.0 Hz, ArH), 8.35 (brs, 1H, NH). ESI-MS: m/z 385 $[M+H]^+$.

2,7,7'-Trimethyl-5-oxo-4-(4-hydroxyphenyl)-1,4,5,6,7,8-hexahydroquinoline-3-carboxylic acid ethyl ester (4j): Yield: 88% as colorless solid, m.p. 237-239 °C. IR (KBr): υ 3416, 1686 cm^{-1}. ^1H NMR ($CDCl_3$+DMSO-d_6): δ 0.91 (s, 3H, CH_3), 1.08 (s, 3H, CH_3), 1.22 (t, 3H, *J* = 7.0 Hz, CH_3), 2.10 (dd, 2H, -CH_2-), 2.30 (s, 5H, CH_3 and CH_2), 4.01 (q, 2H, *J* = 7.0 Hz, -OCH_2-), 4.80 (s, 1H, CH), 6.55 (d, 2H, *J* = 8.5 Hz, ArH), 7.00 (d, 2H, *J* = 8.5 Hz, ArH), 8.30 (s, 1H, OH), 8.43 (brs, 1H, NH). ESI-MS: m/z 356 $[M+H]^+$.

2,7,7'-Trimethyl-5-oxo-4-(4-bromophenyl)-1,4,5,6,7,8-hexahydroquinoline-3-carboxylic acid ethyl ester (4k): Yield: 83%) as crystalline solid, m.p. 250-252 °C. IR (KBr): υ 3275, 1703 cm^{-1}. ^1H NMR ($CDCl_3$+DMSO-d_6): δ 0.90 (s, 3H, CH_3), 1.05 (s, 3H, CH_3), 1.18 (t, 3H, *J* = 7.3 Hz, CH_3), 2.11 (s, 5H, CH_3 and CH_2), 2.30 (s, 2H, -CH_2-), 4.00 (q, 2H, *J* = 7.3 Hz, -OCH_2-), 4.85 (s, 1H, CH), 7.10 (d, 2H, *J* = 8.2 Hz, ArH), 7.22 (d, 2H, *J* = 8.2 Hz, ArH), 8.50 (brs, 1H, NH). ESI-MS: m/z 419 $[M+H]^+$.

2,7,7'-Trimethyl-5-oxo-4-(3,4,5-trimethoxyphenyl)-1,4,5,6,7,8-hydro

quinoline-3-carboxylic acid ethyl ester (4l): Yield: 96% as colorless solid, m.p. 238-240 °C. IR (KBr): υ 3284, 1683 cm^{-1}. ^1H NMR ($CDCl_3$+DMSO-d_6): 0.98 (s, 3H, CH_3), 1.10 (s, 3H, CH_3), 1.25 (t, 3H, *J* = 7.0 Hz, CH_3), 2.12 (s, 5H, CH_3 and CH_2), 2.32 (s, 2H, -CH_2-), 3.68 (s, 3H, OCH_3), 3.76 (s, 6H, 2 x OCH_3), 4.05 (q, 2H, *J* = 7.0 Hz, -OCH_2-), 4.85 (s, 1H, CH), 6.45 (s, 2H, ArH), 8.60 (brs, 1H, NH). ESI-MS: m/z 430 $[M+H]^+$.

2,7,7'-Trimethyl-5-oxo-4-(2,4-dimethoxyphenyl)-1,4,5,6,7,8-hydro

quinoline-3-carboxylic acid ethyl ester (4m): Yield: 87% as cream colored solid, m.p. 268-270 °C. IR (KBr): υ 3289, 1695 cm^{-1}. ^1H NMR ($CDCl_3$+DMSO-d_6): δ 0.92 (s, 3H, CH_3), 1.10 (s, 3H, CH_3), 1.20 (t, 3H, *J* = 7.0 Hz, CH_3), 2.05 (dd, 2H, -CH_2-), 2.28 (s, 5H, CH_3 and CH_2), 3.75 (2s, 6H, 2 x OCH_3), 4.00 (q, 2H, *J* = 7.0 Hz, -OCH_2-), 5.00 (s, 1H, CH), 6.30 (m, 2H, ArH), 7.04 (s, 1H, ArH), 8.60 (brs, 1H, NH). ESI-MS: m/z 400 $[M+H]^+$.

2,7,7'-Trimethyl-5-oxo-4-(4,5-dihydroxy-3-methoxyphenyl)-1,4,5,6,7,8-hexahydro quinoline-3-carboxylic acid ethyl ester (4n): Yield: 88% as light brown colored solid, m.p. 275-277 °C. IR (KBr): υ 3309, 1692 cm^{-1}. ^1H NMR ($CDCl_3$+DMSO-d_6): δ 0.95 (s, 3H, CH_3), 1.00 (s, 3H, CH_3), 1.24 (t, 3H, *J* = 7.3 Hz, CH_3), 2.10 (dd, 2H, -CH_2-), 2.30 (s, 5H, CH_3 and CH_2), 3.78 (s, 3H, OCH_3), 4.04 (q, 2H, *J* = 7.3 Hz, -OCH_2-), 4.78 (s, 1H, CH), 6.30 (s, 1H, ArH), 6.40 (s, 1H, ArH), 7.70 (s, 1H, -OH), 8.45 (brs, 1H, NH). ESI-MS: m/z 402 $[M+H]^+$.

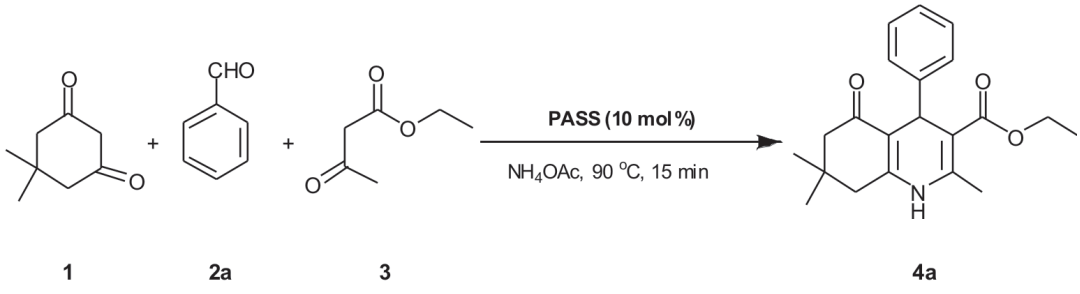
2,7,7'-Trimethyl-5-oxo-4-(4,-*N*,*N*-dimethylphenyl)-1,4,5,6,7,8-hydro

quinoline-3-carboxylic acid ethyl ester (4o): Yield: 80% as cream colored solid, m.p. 263-265 °C. IR (KBr): υ 3279, 1606 cm^{-1}. ^1H NMR ($CDCl_3$+DMSO-d_6): δ 0.90 (s, 3H, CH_3), 1.10 (s, 3H, CH_3), 1.20 (t, 3H, *J* =7.2 Hz, CH_3), 2.10 (dd, 2H, -CH_2-), 2.30 (s, 5H, CH_3 and CH_2), 2.90 (s, 6H, 2 X CH_3), 4.00 (q, 2H, *J* = 7.2 Hz, -OCH_2-), 4.80 (s, 1H, CH), 6.65 (d, 2H, *J* = 7.9 Hz, ArH), 7.10 (d, 2H, *J* = 7.9 Hz, ArH), 8.6 (brs, 1H, NH). ESI-MS: m/z 383 $[M+H]^+$.

RESULTS AND DISCUSSION

We first investigated Hantzsch synthesis for polyhydroquinolines under solvent-free conditions. Thus a mixture of benzaldehyde (**2a**, 0.21 g, 2.0 mmol), 5,5-dimethyl-1,3-cyclohexanedione (**1**, 0.28 g, 2.0 mmol), ethyl acetacetate (**3**, 0.26 g, 2.0 mmol), ammonium acetate (0.23 g, 3.0 mmol) were heated at 90 °C in the presence of a catalytic amount of PASS (10 mol%) for a period of 15 min to afford **4a** (0.60 g, 90%) (**Scheme 2**) as yellow crystalline solid, m.p. 230-232 °C. ESI MS: 340 $[M+H]^+$.

In the ^1H NMR spectrum of compound **4a** methyl protons of cyclohexane ring appeared at δ 0.85 and 1.03 (2s, 6H), ester methyl protons at δ 1.14 (t, 3H, *J* = 7.2 Hz), C-2 methyl and C-8 methylene protons at δ 2.26 (s, 5H), characteristic H-4 proton of DHP ring at δ 4.86 (s, 1H), C-6 methylene group at δ 2.05 (dd, 2H), ester methylene protons at δ 3.96 (q, 2H, *J* = 7.2 Hz), aromatic protons at δ 7.10 (m, 5H), and –NH proton at δ 8.56 (brs, 1H).



Scheme 2

We continued to investigate the reaction using several aromatic aldehydes **2b-o** with 5,5-dimethyl-1,3-cyclohexanedione (**1**), ethyl acetoacetate (**3**) and ammonium acetate at temperature of 90 °C under solvent-free media with 10 mol% PASS to isolate cyclic 1,4-DHPs **4b-o** in yields 85-96% (**Scheme 1**).

Conclusion

In conclusion, the present study describes a convenient and efficient process for the synthesis of polyhydroquinoline derivatives through a four component coupling of various aromatic aldehydes, 5,5-dimethyl-1,3-cyclohexanedione, ethyl acetoacetate and ammonium acetate under solvent-free media at 90 °C using PASS (10 mol %) as a heterogeneous catalyst. Present methodology offers very attractive features such as reduced reaction times, higher yields, etc. This simple procedure combined with recovery and reusability of the catalyst makes this method economic and a waste-free chemical process for the synthesis of polyhydroquinoline derivatives.

References

1. A. Hantzsch, *Ann.,* **1882**, *1*, 215.
2. U. Eisner, J. Kuthan, *Chem., Rev.,* **1972**, *72*, 1.
3. D. M. Stout, A. J. Meyers, *Chem., Rev.,* **1982**, *82*, 223.
4. R. Mannhold, B. Jablonka, W. Voigt, K. Schonafinger, E. Schraven, *Eur. J. Med., Chem.,* **1992**, *27*, 229.
5. J. G. Breitenbucher, G. Figliozzi, *Tetrahedron Lett.,* **2000**, *41*, 4311.
6. L. Ohberg, J. Westman, *Synlett.*, **2001**, 1296.
7. S-J. Ji, Z-Q. Jiang, J. Lu, T-P. Loh, *Synlett*, **2004**, 831.
8. N. Tewari, N. Dwivedi, R. P. Tripathi, *Tetrahedron Lett.,* **2004**, *45,* 9011.
9. L-M. Wang, J. Sheng, L. Zhang, J-W. Han, Z-Y. Fan, H. Tian, C-T. Qian, *Tetrahedron.* **2005**, *61*, 1539.
10. S. Ko, M. V. N. Sastry, C. Li, C-F. Yao, *Tetrahedron Lett.,* **2005**, *46*, 5771.

INTERNATIONAL SCIENCE-TECH RESEARCH INSTITUTE

(RESEARCH, TRAINING AND CONSULTANCY)

- Centre for Food Science & Technology
- Centere for Biotechnology
- Centre for Energy & Environment
- Centre for Natural Products & Herbal Medicines
- Centre for Synthetic and Medicinal Chemistry
- Centre for Pharmacology & Toxicology
- Centre for Sustainable Technologies and Rural Development
- Centre for Enterpreneurship and Livelihood Development
- Centre for Material Sciences & Nano Technology
- Centre for Agriculture & Allid Sciences and Technology
- Center for Coumputer Science & Information Technology

WE UNDERTAKE UG, PG AND OUTSOURCING PROJECTS IN THE ABOVE FIELDS.

HEAD OFFICE

Flat#105, First Floor, Block No: 1,
Radiant Jasmine Gardens, Sy. No: 26/29, Shivana Halli,
Jakkur Road, Yela Hanka, Bangalore-560064.

BRANCH OFFICE

Plot No: 22, Vidyuth Nagar, Anantapur.

ORJAY INFOTECH Pvt. Ltd.

WE UNDERTAKE FOLLOWING WORKS FOR PRIVATE INSTITUTIONS AND ORGANISATIONS

- *Software Solutions*
- *Hardware Solutions*
- *Web Design & Hosters*
- *Publications*
- *Computer and Information Technology Education & Training*
- *Placements*

HEAD OFFICE

2nd FLOOR, # 16 KANKA NAGAR 1st CROSS,
SULTAN PALYA, R.T. NAGAR (P),
BANGALORE – 560032
KARNATAKA
Phone No: +919441489324

BRANCHES:

1. Leela Kumar Chitla, S.No 54,2nd Floor, Telugu Vasti, Mundhwa, Near Old Canal, B.T.Kavadi Road, Ghorpodi, Pune-411036. Mob: +919921694256.
2. Plot No : 22, D.No: 12/3/925, Vidhyuth Nagar, Anantapur, Andhra Pradesh. Mob: +919441489324.
3. Flat No: 401, House No: 16/2/701/8/B, Vijaya Sri Arcade, Ananda Nagar, Malakpet, Hydeabad-36. Mob: +919949394789.

Science – Tech Foundation

Journal of Pharmacy and Chemistry

(An International Research Journal of Pharmaceutical and Chemical Sciences)
(Indexing in CAS)

Plot No. 22, Vidyut Nagar, Anantapur – 515 001(A.P), INDIA

MEMBERSHIP FORM

Dear Editor,

I/We wish to be Annual Member / Life Member of Journal of Pharmacy and Chemistry (An International Research Journal of Pharmaceutical and Chemical Sciences) and agree to abide by your rules and regulations

1. Name in full: _____ Male ☐ Female ☐
   (In block letters)

2. Nationality: _____ 3. Date of birth:_____

3. Corresponding Address: _____

   _____

   _____

5. Institutional / Business Address: _____

6. Phone : (O): _____ (R): _____ E-mail :_____

7. Academic Qualifications : _____

8. Profession and Experience : _____

9. Present Position / Post : _____

10. Pharmaceutical / Chemical interest: _____

Membership Fees	**Life**	**Annual**
Individual	Rs. 8000/- US $ 800	Rs. 2000/- US $ 150
Institutional	Rs. 20,000/- US $ 2000	Rs. 2500/- US $ 200

Rs. / $ _____ remitted by Bank / Cash

DD No. _____ Date _____ Banker's Name_____

DD's are drawn in favour of **Science-Tech Foundation**, Payable at **Anantapur.**

Place: _____

Date: _____

(Signature of the applicant)

Note: (i) All authors should be member of this Journal, (ii) Saperate Photostat copy may be filled and send.

INSTRUCTION TO AUTHORS

GENERAL REQUIREMENTS: Journal of Pharmacy and Chemistry (ISSN 0973-9874) is a quarterly Journal, ***Indexing in CAS(Coden:JPCOCM)*** which publishes original research work that contributes significantly to further the scientific knowledge in Pharmaceutical Sciences (Pharmaceutical Technology, Pharmaceutics, Biopharmaceutics, Pharmacokinetics, Pharmaceutical Chemistry, Computational Chemistry and Molecular Drug Design, Pharmacognosy and Phytochemistry, Pharmacology, Pharmaceutical Analysis, Pharmacy Practice, Clinical and Hospital Pharmacy, Cell Biology, Genomics and Proteomics, Pharmacogenomics, Stem Cell Research, Vaccines & Cera, Bioinformatics and Biotechnology of Pharmaceutical Interest) and in Chemical Sciences (Inorganic, Soil, Forensic, Analytical, Nano, Environmental, Polymer, Physical, Agricultural, Medicinal, Biochemistry, Organic, Computational, Food, Pesticides etc). Manuscripts are accepted for consideration by Journal of Pharmacy and Chemistry on the condition that they represent original material, have not been published previously, are not being considered for publication elsewhere, and have been approved by each author. Review articles, research papers, short communication and letters to the editor may be submitted for publication.

SUBMISSION OF MANUSCRIPTS: Typewritten manuscripts prepared using MS Word should be submitted in triplicate and RW-CD to Prof. Dr. K.N Jayaveera, Editor-in-Chief of Journal of Pharmacy and Chemistry , Plot No 22, Vidyut Nagar, Ananthapur- 515 001, Andhra Pradesh, India. e-mail: jpcanantapur@gmail.com

All components of the manuscript must appear within a single electronic file: references, figure legends and tables must appear in the body of the manuscript.

TYPING INSTRUCTION: The following detailed instructions are necessary to allow direct reproduction of the manuscript for rapid publishing. If instructions are not followed, the manuscript will be returned for retyping. The following typefaces, in 12 points size, are preferred: Times Roman.

GENERAL FORMAT: The typing area must be exactly 6 5/8" (168 mm) wide by 9 7/8" (250 mm) long. Justify margins left and right (block format). The entire typing area of each page must be filled, leaving no wasted space. Text should be double-spaced, special care should be taken to insure that symbols, superscripts and subscripts are legible and do not overlap onto lines above or below. Make sure text lines are equidistant.

TITLE: On the first page of the manuscript, start title 1" (25 mm) down from top text margin. Type title in all capital letters, centred on the width of the typing area and single-spaced if more than one line is required. The title should be brief, descriptive and have all words spelled out. Double-space, then type the author(s) name(s), single-spaced if more than one line is required. Double-space, than type author(s) address(es), single-spaced, capitalizing first letters of main words. Quadruple-space before Abstract.

ABSTRACT: Centre, type and underline abstract heading, capitalizing the first letter. A double-space should separate the heading from the abstract text. Indent abstract text approximately 1/2" (13 mm) from both left and right margins. The abstract should be intelligible to the reader without reference to the body of the paper and be suitable for reproduction by abstracting services. Introduction to the text (without a heading) should being four spaces below the abstract using full margins.

KEY WORDS: Three or more key words must be provided by authors for indexing of their article. Key words will be listed directly below the Abstract. Abbreviated forms of chemical compounds are not acceptable. Spell out entirely, using the official nomenclature. Example: L-dihydroxyphenylalanine (L-DOPA)

MAJOR HEADINGS: Papers must include the major headings: Introduction, Methods, Results, Discussion, Acknowledgments and References. Capitalize first letter, underline, and centre headings on width of typing area.

TABLES/FIGURES: Incorporate tables and/or figures (B & W) with their legends into the main body of text.

REFERENCES: Referneces should be referred to a number [1] in the text and be listed according to this numbering at the end of the paper. Only papers and books that have been published or in press may be cited; unpublished manuscripts or manuscripts submitted to a journal but which have not been accepted may not be cited.

The references shoulld comprise the following information and in the given order and with given punctuation as given in the example below: Author name (s), Initials (s), Publication Title, Page Number, Year of Publication.

Standard Journal Article:

[1] Bhattacharyya D, Pandit S, Mukherjee R, Das N, Sur TK. Indian J Physiol Pharmacol 2003; 47:435.

[2] Skottova N, Krecman V. Physiol Res 1998; 47:1.

Book:

[1] Ghosh MN. Fundamentals of Experimental Pharmacology, 2nd ed. Calcutta Scientific Book Agency, 1984:154.

Proofs will be sent to the corresponding author. These should be returned as quickly as possible.

The facts and view in the article will be of the authors and they will be totally responsible for authenticity, validity and originality etc. the authors should give an undertaking while submitting the paper that the manuscripts submitted to journal have not been published and have not been simultaneously submitted or published elsewhere and manuscripts are their original work.

www.jpc.stfindia.com
www.stfindia.com

This Quarterly Journal is Printed, Published, Owned and Edited by Dr. K.N. Jayaveera, Published from Science-Tech Foundation, Plot No.22, Door No. 12/3/925, Vidyut Nagar, Anantapur - 515 001. Send your queries at www.stfindia.com and Printed at Vipla Computer Services (Designers & Multi Colour Offset Printers) # 2-3-720/1A/22/40, Ganga Nagar, Opp. Govt. Primary School, Zinda Tilismat Road, Golnaka, Amberpet, Hyderabad - 500 013. Ph. 040-27426910, 27427078.