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ARTICLES

Research Articles

Review

A review on novel therapies to combat hepatitis C

Pragati Khare*, Lubhan Singh and Ghanshyam Yadav

Department of Pharmaceutical Technology, Meerut Institute of Engineering and Technology, NH-58, Bagpat Crossing, Bypass Road, Meerut, U.P.-250005, India.

The major cause of chronic liver disease, cirrhosis, liver carcinoma and liver failure is Hepatitis C virus (HCV). NS3 is one of the attractive targets for therapy development for HCV as the N-terminal domain of the NS3 protein is a serine protein. NS3 protease acts by interfering with cellular mechanisms which are involved in the host immune response to an HCV infection and the inhibition of the protease is an efficient antiviral approach. NS3 protease inhibitors initially developed were: BILN 2061 (Ciluprevir), VX-950 (Telaprevir) and SCH503034. A combination therapy of injected pegylated interferon-α and oral ribavirin is regarded as the current standard therapy for HCV infection. Leucopenia, flu-like symptoms, thrombocytopenia, depression and anemia are some of the adverse effects of ribavirin. The compounds which constitute a benzoxaborole moiety interact with many biological targets and presents appreciable qualities. The acyl sulfinamide and the acyl cyanamide were shown to be potent P1 C-terminal groups in the enzyme assay. In a study, it is indicated that the aminobenzoyl sulfonamide fragment was identified as a novel P1 structural motif. New P2–P4 macrocycle inhibitors of NS3/4A were made up of a P1 C-terminal carboxylic acid have recently been developed. Potent tetrapeptidic inhibitors of the HCV NS3 protease have been developed by incorporating 4-hydroxy-cyclopent-2-ene-1,2-dicarboxylic acid as a new N-acyl-L-hydroxyproline were developed for the treatment of Hepatitis C. α-Amino cyclic boronates was designed and synthesized and incorporated successfully in several acyclic templates at the P1 position as HCV inhibitors. The boronic acid compounds acts as the HCV NS3 serine protease inhibitors. Some of the HCV-protease inhibitors are developed which are based on a 2(1H)-pyrazinone P3 scaffold in combination with either a P2 phenylglycine or a glycine. NS3/4A protease inhibitors constituting of quinazoline derivatives as P2 substituent were synthesized. The tripeptide-based inhibitors of the HCV NS3 protease containing a novel P2-triazole had been synthesized. A protease domain (NS3pro) and RNA helicase domain (NS3hel) makes a multifunctional enzyme: Flaviviridae non-structural 3 proteins (NS3). Crude ethanol extract from rhizomes of the Chinese medicinal herb *Rhodiola kirilowii* (Regel) Maxim was also used for the cure of Hepatitis C. A new series of HCV NS3/4A protease inhibitors bearing a P2-P4 macrocycle and a P1- P10 a-ketoamide serine trap has been studied.

Key words: Hepatitis C virus, non-structural protein inhibitors.

INTRODUCTION

"Hepatitis" means inflammation of the liver. Hepatitis is most often caused by a virus. The most common types of viral hepatitis are hepatitis A, hepatitis B and hepatitis C. Heavy alcohol use, toxins, some medications, and certain medical conditions can also cause hepatitis. Hepatitis

may be of two types:

1. Acute hepatitis C is a short-term illness that occurs within the first 6 months after someone is exposed to the HCV.

2. Chronic hepatitis C is a long-term illness that occurs when the HCV remains in a person's body.

It can lead to serious liver problems, including liver damage, cirrhosis, liver failure and liver cancer. Since the characterization of the HCV, from last 15 years, the understanding of the natural history of chronic hepatitis C has been greatly expanded and more effective and useful therapeutic strategies have been developed. Sustained virological response (SVR) rates have been improved from > 20% of patients treated for 48 weeks with conventional interferon (IFN) alfa, to ≈ 40% of patients treated with the combination of IFN alfa-2b plus ribavirin, and 54 to 61% of patients treated with a pegylated IFN and ribavirin, the current treatment of choice. Regulatory T cell markers are increased in chronically infected individuals with the HCV. The naturally occurring viral variants inhibit the T cell responses to cognate NS3358−375 in an antigen-specific manner (Duan et al., 2004). Hepatitis C is usually spread when blood from a person infected with the hepatitis C virus enters the body of someone who is not infected. Most people become infected with hepatitis C by sharing needles or other equipment to inject drugs. Hepatitis C was also commonly spread through blood transfusions and organ transplants. Symptoms for both acute and chronic hepatitis C can include fever, fatigue, loss of appetite, nausea, vomiting, abdominal pain, dark urine, grey-colored stools, joint pain and jaundice.

Diagnosis of hepatitis C

The diagnosis of hepatitis C can be done by specific blood tests. A person first gets a screening test that looks for "antibodies" to the HCV. The antibodies remain in the bloodstream, even if the person clears the virus.

Prevention of hepatitis

1. Do not share needles or other equipment to inject cosmetic substances, drugs, or steroids.
2. Do not use personal items that may have come into contact with an infected person's blood, such as razors, nail clippers, toothbrushes, or glucose monitors.
3. Do not get tattoos or body piercings from an unlicensed facility or in an informal setting (hepc general fact sheet).
   S370P induced regulatory T cell markers in comparison to NS3358−375-stimulated CD4 T cells. The HCV is able to induce antigen-specific regulatory T cells to suppress

the antiviral T cell response in an antigen-specific manner. The variants of an HCV immunodominant epitope, which develop during the chronic infection in a human, induced Foxp3 expression in an antigen-specific manner. It had a dose-dependent suppressive effect *in vitro*. The major cause of chronic liver disease is HCV. HCV causes cirrhosis, liver carcinoma and liver failure, and ultimately liver transplants. About 200 million people have been infected chronically with HCV (Xianfeng et al., 2010). HCV genome is a single- stranded, positive sense RNA molecule of 9600 nucleotides (Ronn et al., 2007). HCV genome is 9.6 kb and the structural proteins C, E1, E2 and non-structural proteins NS2, NS3, NS4A, NS4B, NS5A and NS5B are encoded by HCV genome. NS3 is one of the attractive targets for therapy development for HCV as the N-terminal domain of the NS3 protein is a serine protein (Duan et al., 2004). The NS3 protein is basically a bifunctional enzyme with a helicase/NTPase domain and a protease domain. NS3 protease acts by interfering with cellular mechanisms which are involved in the host immune response to an HCV infection. The inhibition of the protease is an efficient antiviral approach that will block viral replication and will also restore the host immune response. NS3 protease inhibitors to enter research were: BILN 2061 (Ciluprevir), VX-950 (Telaprevir) and SCH503034. Telaprevir and SCH 503034 are being used in US as useful remedies to treat HCV-infected patients but the clinical evaluation of Ciluprevir was stopped because of cardiac toxicity in animal model (Ronn et al., 2007).

   A combination therapy of injected pegylated interferon-α and oral ribavirin is regarded as the current standard of care for HCV infection, leukopenia, flu-like symptoms, thrombocytopenia, depression, anemia are some of the adverse effects of ribavirin (Xianfeng et al., 2010). BILN 2061 is mainly characterized by three unnatural amino acid residues (P1, P2, P3) in a macrocycle. The oral administration of BILN 2061 to the patients of hepatitis C caused an impressive reduction of viral RNA levels showing the proof for HCV NS3/4A protease inhibitors (Raboisson et al., 2008a). NS3/4A is the virally encoded serine protease which is one of the important constituent of life cycle of the HCV and is responsible for chronic hepatitis, liver cirrhosis and hepatocellular carcinoma. The development for potent and selective HCV NS3/4A protease inhibitors is the target for pharmaceutical industry for administering efficacious drugs to cure HCV – infected patients (Raboisson et al., 2008b).

NOVEL THERAPIES FOR HEPATITIS C

HCV is responsible for infecting approximately 3% of

*Corresponding author. E-mail: pragatikhare10@gmail.com. Tel: 9412372249, +91-121-2439057 ext: 2439019. Fax: +91-121-2439058.

world's population. It has been observed that the compounds which constitute a benzoxaborole moiety interacts with many biological targets and shows appreciable drug qualities. The benzoxaborole moiety can potentially form polar interactions with Thr 42 and positively charged Lys 136. Studies have been performed on the inhibitory potency of different regioisomers of acylsulfamoyl benzoxaboroles-based HCV NS3 protease inhibitors. Appropriate optimization of the benzoxaborole moiety may rebalance the physicochemical properties of the resulting compounds and enhance their membrane absorption, potency and bioavailability (Xianfeng et al., 2010). The major problem in the use of antiviral drugs is the development of drug resistance. Mutations causing resistance to BILN-2061, VX-950, and SCH 503034 have been observed in the subgenomic HCV replicon assay. The acyl sulfinamide and the acyl cyanamide were shown to be potent P1 C-terminal groups, in the enzyme assay (Ro¨nn et al., 2007).

In a study, it is indicated that the aminobenzoyl sulfonamide fragment was identified as a novel therapy for hepatitis C. A microwave irradiated, palladium catalyzed, amidocarbonylation protocol caused the facile preparation of a series of compounds depicting sub-micromolar potencies in the full-length NS3 assay. According to the study, the inhibitors have two sites that can be used in optimization process (Ro¨nn et al., 2008). HCV–NS3 protease is necessary for viral replication hence; NS3 protease inhibitors have proved to be efficient targets in clinical trials. A series of analogs were developed in which the carboxylic residue is replaced by phosphorous acid functionalities and worked as the inhibitors of the NS3 protease. The methylphosphinate analogue showed nanomolar level of enzyme inhibition and sub-micromolar potency in the replication assay. A novel class of P2–P4 macrocycle analogues acted as the inhibitors of HCV–NS3 protease, MK-7009; a potent inhibitor bearing a cyclopropylacylsulfonamide in P1 showed strong antiviral activity in HCV infected chimpanzees and is currently being evaluated in clinical trials (Pompei et al., 2009).

For the treatment of hepatitis C, potent tetrapeptidic inhibitors of the HCV NS3 protease have been developed by incorporating 4-hydroxy-cyclopent-2-ene-1,2-di-carboxylic acid as a new N-acyl-L-hydroxyproline. The hydroxylcyclopentene template was synthesized from commercially available (syn)-tetrahydrophthalic anhydride. Three different amino acids were discovered in the P1-position, and in the P2-position the hydroxyl group of the cyclopentene template was substituted with 7-methoxy-2-phenyl-quinolin-4-ol. The P3/P4-positions were optimized from a set of six amino acid derivatives. All inhibitors were evaluated in an *in vitro* assay using the full-length NS3 protease. Several potent inhibitors were identified, with the most promising exhibiting a Ki value of 1.1 nM (Thorstensson et al., 2007).

There are many potent novel HCV NS3 protease inhibitors which have been developed from two inhibitor series constituting either a P2 trisubstituted macrocyclic cyclopentane- or a P2 cyclopentene dicarboxylic acid moiety as surrogates for the widely used Nacyl-(4R)-hydroxyproline in the P2 position. According to the studies, it was found that the 14-membered ring system had a better potency in these two series and that the corresponding 13-, 15-, and 16-membered macrocyclic rings exhibited lesser potency. It has been observed that P1 acylsulfonamides had better potencies than the corresponding P1 carboxylic acids. Trisubstituted cyclopentane- and cyclopentene dicarboxylic acid moieties have been used to replace the commonly used N-acyl-(4R)-hydroxyproline, and have been incorporated in the P2 position of macrocyclic functionalized HCV NS3 inhibitors (Back et al., 2007).

The inhibitors of the HCV NS3 serine protease, α-amino cyclic boronates, was designed and synthesized and incorporated them successfully in several acyclic templates at the P1 position. The structural studies depict that they inhibit the NS3 protease by trapping the Ser-139 hydroxyl group at the active site. The first peptidomimetic boronic acid is velcade that has been developed into a therapeutic agent for the cure of relapsed multiple myeloma. The boronic acid compounds acts as the HCV NS3 serine protease inhibitors by many research groups in pharmaceutical companies such as Schering-Plough, Phenomix, Dupont, and BMS (Xianfeng et al., 2010).

A new class of phosphonate derivatives was developed to copy the interaction of product-like carboxylate based inhibitors of HCV NS3 protease. A phosphonic acid was reported to be a potent HCV NS3 protease inhibitor and utilized for the treatment of HCV infection (Sheng et al., 2009).

HCV- protease inhibitors are developed which are based on a 2(1H)-pyrazinone P3 scaffold in combination with either a P2 phenylglycine or a glycine. These HCV-protease inhibitors were further evaluated on the wild type as well as on two resistant variants of the enzyme, A156T and D168V. According to the molecular modeling the aromatic side-chain of the P2 phenylglycine occupies the same space as the substituent in position 6 on the pyrazinone core. The aromatic P1–P10 scaffold was found to be better in combination with the new P3–P2 building block and an entirely new type of achiral and rigidified inhibitors was discovered with enhanced potency. These developed inhibitors may be utilized for further optimization in a combinatorial fashion because of the rapid synthetic scheme for pyrazinone synthesis, the easy access to structurally diverse starting materials for the pyrazinone synthesis, like aldehydes, primary amines and a cyanide source, and the short (five steps) overall synthetic route of the inhibitor synthesis (Nilsson et al., 2010).

NS3/4A protease inhibitors constituting of quinazoline derivatives as P2 substituent were synthesized. By the optimization of quinazoline P2 substituents in three series

having macrocyclic P2 cyclopentane dicarboxylic acid and P2 proline urea motifs, better potency inhibitors depicting valuable pharmacokinetic properties can be obtained. An enhanced metabolic stability in human liver microsomes was obtained by 8-methyl substitution in the P2 cyclopentane dicarboxylic acid series in the quinazoline moiety. The proline urea series exhibited useful CaCO^{-2} permeability than the cyclopentane series. A better pharmacokinetic property was observed *in vivo* in rats (Naud et al., 2008).

The tripeptide-based inhibitors of the HCV NS3 protease containing a novel P2-triazole had been designed and synthesized. A diverse series of inhibitors can be produced by the replacement of the P2 quinoline with a triazole moiety. Improvement may be done by the incorporation of an aryl-substituted triazole and by the replacement of the P1 acid with an acyl sulfonamide. Thus, these inhibitors produced appreciable cellular activity (Yao et al., 1999).

HCV is responsible for infecting approximately 3% of the world's population. HCV RNA is translated into a polyprotein. It gets cleaved into functional components that is, nonstructural protein 3 (NS3), which is a 631-residue bifunctional enzyme with protease and helicase activities. The scNS3–NS4A structure provides the first atomic view of polyprotein *cis* processing. The NS3 serine protease processes the HCV polyprotein by both *cis* and *trans* mechanisms. The local and global structural rearrangements follow the *cis* cleavage reaction, and large segments of the polyprotein can be folded prior to proteolytic processing. The product complex of the *cis* cleavage reaction exists in a stable molecular conformation and suggests autoinhibition and substrate-induced activation mechanisms for the regulation of NS3 protease activity (Xiaoping et al., 2009).

HCV protease inhibitors targets HCV NS3 which can effectively suppress HCV replication. Luciferase-reporter 1b replicon shuttle vectors allow the cloning of either the HCV full-length NS3/4A gene or the NS3 protease domain gene which only were developed. Initially, chimeric replicons carrying patient derived full-length NS3/4A failed to replicate in cell culture but the poor replication efficiency of the NS3/4A shuttle vector was enhanced by approximately 100-fold when the NS3 helicase domains of clinical isolates were substituted for that of the 1b Con1 lab strain. The HCV replicon system has been utilized to study the potency and mechanism of HCV inhibitors in a cell-based format. The current subgenomic or full-length genomic replicons are established with laboratory-optimized strains. According to the studies, shuttle vector approach may be used for the NS5B gene encoding the RdRp in which the NS5B gene was isolated from the sera of HCV-infected patients and then shuttled to a replicon vector deficient in RdRp activity to restore the RNA replication. A good potency was observed for the inhibitor when the replicons containing patient-derived NS5B from a panel of clinical

isolates were tested against a polymerase inhibitor. Hence, this approach is useful for the evaluation of the phenotype of a mixed quasispecies pool. The full-length NS3/4A and NS3 protease domain are useful for the novel therapy of hepatitis C (Salloumia et al., 2010). The mutations causing resistance may reduce the efficiency of NS3/4A serine protease. The level of resistance associated with specific mutations differs from compounds to compounds. The substitutions R155K and A156T reduce the activity of all protease inhibitors. The R155K variant is having a high replication level and there is a substantial loss of cross-recognition by specific CD8 T cells targeting the epitope HAVGIFRAAV1175–1184 (Bogen et al., 2005).

The replication of the HCV takes place by the help of HCV NS3 protease and NS5B polymerase, a number of competitive inhibitors of the NS3 protease as well as nucleoside and non-nucleoside inhibitors of the NS5B polymerase have been identified by combining the power of high-throughput screening with rational, knowledge-based drug discovery. The HCV NS3 protein is a component of a heterodimeric serine protease that requires the noncovalently-associated viral protein NS4A for better catalytic activity. NS3-4A proteinase enzyme is responsible to cause the proteolytic cleavage of the viral polyprotein at the four junctions NS3/NS4A, NS5A/NS5B, NS4A/NS4B and NS4B/NS5A. NS3-4A protease plays an important role in the production of the viral RNA replication machinery by mediating a number of events in the proteolytic processing and maturation of the nonstructural viral proteins. By the help of this enzyme, the virus antagonizes the host cell innate immune response. NS3-4A causes the cleavage of CARDIF and TRIF which are the two essential components by which the cells sense the invasion by viral pathogens thus causing the induction of the antiviral state. NS3-4A counteracts the onset of the cell natural antiviral defenses. NS3-4A inhibitor prevents polyprotein processing and restores the hepatocytes innate antiviral response, thus depicting dual antiviral activity (Francesco et al., 2007).

A protease domain (NS3pro) and an RNA helicase domain (NS3hel) makes a multifunctional enzyme: Flaviviridae non-structural 3 protein (NS3). The activities of NS3 are critical for the viral replication. The replicative cycle of Flaviviridae needs the coordinated regulation of all the activities of NS3 protein. By the analysis of the root mean square (RMS) variation, NS3pro increases the stability of the subdomain 1 of the RNA helicase. By the utilization of the normal mode analysis, studies characterized slow collective motions of NS3, and observed that the two lowest-frequency normal modes are enough to represent reorientations of NS3pro relative to NS3hel. These movements induced an enhancement in the exposure of the active site of NS3pro that can be important during the proteolytic processing of the viral polyprotein (Rosales-Leon et al., 2007). To develop the

naturally occuring chemical entities as lead compounds from which the novel anti-HCV agents could be developed, the bioassay-guided fractionation and isolation were performed on a crude ethanol extract from rhizomes of the Chinese medicinal herb *Rhodiola kirilowii* (Regel) Maxim by the use of column chromatography (CC) techniques and *in vitro* inhibitory activity against HCV NS3-SP. The partition of the extract between water and different organic solvents caused the isolation and identification of 12 compounds in the ethyl acetate part which was the most active. The compounds were tested for *in vitro* activity against HCV NS3-SP, and the most potent ones are: (−)-Epicatechin, derivatives: 3,3_-digalloylproprodelphinidin B2 (Rhodisin); 3,3_-digalloylprocyanidin B2; (−)-epigallocatechin-3-O-gallate; and (−)-epicatechin-3-O-gallate with IC_{50} of 0.77, 0.91, 8.51 and 18.55 _M, respectively (Zuoa et al., 2007).

A new series of HCV NS3/4A protease inhibitors bearing a P2-P4 macrocycle and a P1- P10 a-ketoamide serine trap has been studied. The NS3 protease which is important for the viral replication has been proved to be one of the attractive targets for developing novel anti-HCV therapies. The optimization of macrocycle led to the discovery of compounds 8b and 8g with a good activity both in the enzyme as well as in the cell based (replicon) assays with favorable PK profile in a preclinical species (Avolio et al., 2009).

The persons who must be tested for hepatitis c

Patients who should proactively be offered testing for hepatitis C include:

1. Haemodialysis patients.
2. Blood, blood product, tissue and organ donors.
3. All drug users, especially those who have injected drugs or shared 'works', including prisoners.
4. Babies born to hepatitis C infected mothers.
5. Immigrants from countries of high endemicity for hepatitis C infection.
6. Persons, including healthcare workers, who have had potential percutaneous or mucous membrane exposure to hepatitis C.
7. HIV or hepatitis B infected patients.
8. Those with unexplained persistently raised serum transaminases.

Hepatitis C testing should be considered on:

1. Sexual partners of people who have hepatitis C (low risk).
2. Men who have sex with men who present for sexually transmitted disease screening (low risk).
3. Those with tattoos or body piercing.

Laboratory methods for hepatitis c testing

Screening for hepatitis C includes testing of blood for the presence of HCV antibodies or hepatitis C antigen in serum. Initially, enzyme immunoassay (EIA) test was utilized for the screening of hepatitis C virus. Acute, chronic or resolved infection can be identified by antibody and antigen testing. The molecular investigation includes the detection of hepatitis C RNA, hepatitis C genotype testing and estimation of the viral load. Genotyping may be used for the detection of HCV (National Hepatitis C Strategy, 2011).

CONCLUSION

About 200 million people have been infected chronically with HCV. NS3 is one of the attractive targets for therapy development for HCV as the N-terminal domain of the NS3 protein is a serine protein. Various therapies have been developed for the treatment of hepatitis C and some are currently under clinical trials. BILN 2061 (Ciluprevir), VX-950 (Telaprevir) and SCH503034 are some of the compounds which were developed initially. A combination of pegylated interferon-α and oral ribavirin compounds constituting of benzoxaborole moiety, acyl sulfinamide and acyl cyanamide, α-amino cyclic boronates, boronic acid compounds, NS3/4A protease inhibitors constituting of quinazoline derivatives, crude ethanol extract from rhizomes of the Chinese medicinal herb *Rhodiola kirilowii* (Regel) Maxim are some of the therapies against hepatitis C. By utilizing different approaches and by molecular modification of the existing drug molecules, hepatitis C may be treated efficiently.

Abbreviations:

NS3, Non-structural protease; **HCV,** hepatitis C virus.

REFERENCES

Avolio S, Robertson K, Hernando JIM, DiMuzio J, Summaa V (2009). Inhibitors of hepatitis C virus NS3/4A: a-Ketoamide based macrocyclic inhibitors. Bioorg. Med. Chem. 19:2295-2298.

Bäck M, Johansson PO, Wångsell F, Thorstensson F, Kvarnström I, Ayesa S, Wähling H, Pelcman M, Jansson K, Lindström S, Wallberg H, Classon B, Rydergård C, Vrang L, Hamelink E, Hallberg A, Rosenquist S, Samuelsson B. (2007). Novel potent macrocyclic inhibitors of the hepatitis C virus NS3 protease: Use of cyclopentane and cyclopentene P2-motifs. Bioorg. Med. Chem. 15:7184-7202.

Bogen S, Saksena AK, Arasappan A, Gu H, Njoroge FG, Girijavallabhan V, Pichardo J, Butkiewicz N, Prongay A, Madison V (2005). Hepatitis C virus NS3-4A serine protease inhibitors: Use of a P2–P1 cyclopropyl alanine combination for improved potency. Bioorg.

Med. Chem. 15:4515-4519.

Duan D, Li Z, Luo H, Zhang W, Chen L, Xu X (2004). Antiviral compounds from traditional Chinese medicines Galla Chinese as inhibitors of HCV NS3 protease. Bioorg. Med. Chem.14:6041-6044.

Francesco RD, Carfí A, Molecolare IDRDB (2007). Advances in the development of new therapeutic agents targeting the NS3-4A serine protease or the NS5B RNA-dependent RNA polymerase of the hepatitis C virus. Adv. Drug Deliv. Rev. 59:1242-1262.

Naud J, Lemke C, Goudreau N (2008). Potent triazolyl-proline-based inhibitors of HCV NS3 protease. Bioorg. Med. Chem. 18:3400-3404.

Nilsson M, Belfrage AK, Lindström S (2010). Synthesis and SAR of potent inhibitors of the Hepatitis C virus NS3/4A protease: Exploration of P2 quinazoline substituents. Bioorg. Med. Chem. 20:4004-4011.

Pompei M, Francesco MED, Koch U, Liverton NJ (2009). Phosphorous acid analogs of novel P2–P4 macrocycles as inhibitors of HCV–NS3 protease. Bioorg. Med. Chem. 19:2574-2578.

Raboisson P, Kock HD, Rosenquist A (2008a). Structure–activity relationship study on a novel series of cyclopentane-containing macrocyclic inhibitors of the hepatitis C virus NS3/4A protease leading to the discovery of TMC435350. Bioorg. Med. Chem. 18:4853-4858.

Raboisson P, Lin TI, Kock HD, Vendeville S (2008b). Discovery of novel potent and selective dipeptide hepatitis C virus NS3/4A serine protease inhibitors. Bioorg. Med. Chem. 18:5095-5100.

Ronn R, Gossas T, Sabnis YA (2007). Evaluation of a diverse set of potential P1 carboxylic acid bioisosteres in hepatitis C virus NS3 protease inhibitors. Bioorg. Med. Chem. 15:4057-4068.

Rosales-Leon LR, Lule GA, Ordaz BR (2007). Analysis of the domain interactions between the protease and helicase of NS3 in dengue and hepatitis C virus. J. Mol. Graph Model. 25:585-594.

Sallouma S, Klugea SF, Kimb AY, Roggendorfa M (2010). The resistance mutation R155K in the NS3/4A protease of hepatitis C virus also leads the virus to escape from HLA-A*68-restricted CD8 T cells. Antiviral Res. 87:272-275.

Thorstensson F, Wångsell F, Kvarnström I, Vrang L, Hamelink E, Jansson K, Hallberg A, Rosenquist S, Samuelsson B (2007). Synthesis of novel potent hepatitis C virus NS3 protease inhibitors: Discovery of 4-hydroxy-cyclopent-2-ene-1, 2-dicarboxylic acid as a N-acyl-L-hydroxyproline bioisostere. Bioorg. Med. Chem. 15:827-838.

Yao N, Reichert P, Taremi SS (1999). Molecular views of viral polyprotein processing revealed by the crystal structure of the hepatitis C virus bifunctional protease–helicase. Structure 7:1353-1363.

Zuoa G, Li Z, Chena L, Xua X (2007). Activity of compounds from Chinese herbal medicine *Rhodiola kirilowii* (Regel) Maxim against HCV NS3 serine protease. Antiviral Res. 76:86-92.

Full Length Research Paper

Determination of the abortifacient activity of the aqueous extract of *Phytolacca dodecandra* (L'Her) leaf in Wistar rats

Angella Namulindwa[1], David Nkwangu[1] and Joseph Oloro[2]*

[1]Department of Pharmacy, Faculty of Medicine, Mbarara University of Science and Technology, P. O. Box 1410, Mbarara, Uganda.
[2]Department of Pharmacology and Therapeutics, Faculty of Medicine, Mbarara University of Science & Technology, P. O. Box 1410, Mbarara, Uganda.

Throughout history women have tried to control their fertility using herbal remedies; traditionally *Phytolacca dodecandra* leaves have been used as an abortifacient. The objective of this study was to determine the abortifacient activity of the aqueous extract of *P. dodecandra* leaf in Wistar rats. Hundred grams of shaded dried powdered leaves were soaked in 0.5 L of cold water, and using cold maceration, an aqueous extract was obtained. Acute toxicity was carried out using Lorke's method. The abortifacient activity of the plant extract was tested using a modified method. Thirty pregnant rats were randomly distributed into five groups each consisting of 6 rats and were treated. The percentage number of rats that aborted per treated group was compared with those of the controls. Preliminary phytochemical screening of the extract revealed the presence of alkaloids, tannins, phenolics, steroids and triterpenoids. Toxicity signs such as reduced appetite, sleepiness, shivering and excessive urination were observed at a dose of 2048 mg/kg; however, no deaths were observed. In the groups in which 125 and 250 mg/kg of *P. dodecandra* extract was administered, 83.3% of the rats aborted, and in the group in which 500 mg/kg was administered, 100% of the rats aborted. This study has substantiated the abortifacient activity of the aqueous extract of *P. dodecandra* leaves which may be attributed to the phytochemicals.

Key words: Abortifacient, phytochemical screening, acute toxicity, *Phytolacca dodecandra*.

INTRODUCTION

Traditional medicine usage in rural Ugandan population for day-to-day healthcare need is close to 90% (Kamatenesi et al., 2006). Despite the limited availability of safety and efficacy information about a number of herbal medicinal preparations, dispensing and use of these products is one of the common health care practices in Uganda (Lutoti et al., 2013). Throughout history, women have tried to control or enhance their fertility. Many herbal remedies are traditionally used as contraceptives (to prevent the ovulation or fertile-zation), abortifacients (to prevent implantation) and emmena-gogues (to prevent uterine flow) or oxytocics (to stimulate

uterine contractions, particularly to promote labour) (Ritchie, 2001). Abortion is the termination of pregnancy by removal or premature expulsion from the uterus of a fetus or embryo prior to viability (Grimes et al., 2006). Abortion may be due to maternal exposure to chemicals, which can disrupt pregnancy and cause detachment of the embryo (Feranada et al., 2000). Currently, the use of herbs to terminate pregnancy by many young women of reproductive age is high yet some of these herbs often need to be used in doses that may be toxic to the woman or even potentially fatal (Selin, 1997; Kress, 2013). Others do not work but rely on the fact that spontaneous abortion is common; in such cases, the occurrence of the abortion is attributed to the herbs if any were being used (Selin, 1997; Kress, 2013).

Traditionally, in Central and Western Uganda, the aqueous extract of *Phytolacca dodecandra* is used to terminate pregnancy within the first six to eight weeks after the last menstrual period (Prada et al., 2005; Selin, 1997). Little information is available on the pharmacological properties and toxicity of abortifacient herbal preparations in Uganda, yet the use of herbs to terminate pregnancy is high and sometimes with serious consequences including death.

Therefore, there is an urgent need to determine and document the abortifacient activity of such herbs and also determine the acute toxicity.

In addition, screening of plants with abortifacient activity and subsequent identification and characterization of the active principle will prove to be useful guide towards the formulation of cheaper, affordable contraceptives with reduced toxicity (Dabhadkar and Zade, 2013).

This therefore provides an avenue for the current study to evaluate the abortifacient activity of the aqueous extract of the leaves of *P. dodecandra*.

P. dodecandra is a climbing or scrambling dioecious, semi-succulent shrub sometimes a liana with glabrous stems up to 10 to 20 m long (Zimudzi, 2007). In traditional medicine, the plant is to treat various ailments such as diarrhea, intestinal infestation, skin infections and dysmenorrhea in humans and animals (Nalule et al., 2011).

The leaf extract of *P. dodecandra* contains terpenoids, phenolics and alkaloids (Ogutu et al., 2012). According to katende et al. (1995), *P. dodecandra* is a very poisonous plant in both people and animals. The leaves, roots and seeds of *Phytolacca acinosa*, a plant from the same family as *P. dodecandra* were found to have abortifacient activity by causing uterine stimulation in rats (Yeung et al., 1987). Therefore, the aim of this study was to determine the abortifacient activity of *P. dodecandra* leaves.

Screening of plants with abortifacient activity and subsequent identification and characterization of the active principle is a useful guide towards the formulationof cheaper, affordable contraceptives with reduced toxicity (Dabhadkar and Zade, 2013).

MATERIALS AND METHODS

The materials and equipment used for carrying out the experiment included 40 female Wistar rats aged three months, *P. dodecandra* leaves, misoprostol tablets (200 mcg), HCG strips, intragastric rubber cannulas, distilled water, steam bath, phytochemical screening reagents of analytical grade, glassware, oven, surgical blades, Analytical balance, blender, Vacuum pump filter, cages and fridge.

Study site

The study was carried out in the pharmaceutical chemistry laboratory, biochemistry laboratory and Animal Research Facility of Mbarara University of Science and Technology.

Study design

This was an experimental short term prospective research study.

Plant collection and extract preparation

Healthy mature plant leaves were collected in January from Wakiso district, Uganda (0.4000°N, 32.4833°E), specimen taken for identification at the Department of Botany, Faculty of Science, Mbarara University of Science and Technology, and was given a Voucher Number: Angella Namulindwa 001. The plant leaves were washed and shade dried for 14 days at room temperature, then pulverized using a blender. 100 g of the leaf powder were soaked in 0.5 L of cold distilled water for 48 h at room temperature with intermittent shaking to allow complete extraction. The mixture was filtered using a vacuum pump filter with Whatman filter paper No. 1 to obtain the filtrate. The filtrate was then concentrated to a constant weight using an oven maintained at temperature of 50°C.

Experimental animals

Female Wistar rats (155 to 280 g) were purchased from the animal facility of Mbarara University of Science and Technology. The animals were initially acclimatized to the laboratory conditions for one week prior to the experiment. The animals were housed in appropriate cages under a 12 h light:dark cycle and were allowed free access to standard rat feeds and clear drinking water *ad libitum*. Animals were handled in this study as per the National Institute of Health guidelines (2011) for the care and use of laboratory animals.

Phytochemical screening

Identification of the chemical constituents of the aqueous leaf extract of *P. dodecandra* was carried out as per standard procedures by Anees and Mohammed (2004).

Acute toxicity studies (LD$_{50}$) using Lorke's method (Lorke, 1983)

The test was performed in one phase. Nine rats were randomly selected, placed in three groups each consisting of three rats. For each group, the rats were treated by gavage with varying dose levels of the reconstituted aqueous extract (800, 1280, and 2048 mg/kg). The treated rats were observed for 6 h after administration of the extract for signs of toxicity such as sleepiness, reduced

Table 1. Phytochemical Screening Results of *P. dodecandra*.

Phytochemical	Test method	Deduction
Alkaloids	Picric acid test	Present
Tannins	Ferric chloride test	Present
Phenols	Ferric chloride test	Present
Reducing sugars	Fehling's test	Present
Steroids and triterpenoids	Libermann-Buchard test	Present
Free amino acids	Ninhydrin test	Present

appetite, shivering and general behavior. The rats were further observed for 14 more days for delayed signs of toxicities and deaths.

Determination of abortifacient activity of *P. dodecandra* leaves

The abortifacient activity of the plant extract was tested in pregnant female rats by modification of the method described by Khanna and Chaudhary (1968). Female rats were caged with males of proven fertility in a ratio of 2:1 in the evening and the following day they were checked for evidence of copulation. This day was considered as day 0 of pregnancy.

After ten days, the rats were tested for pregnancy using HCG strips. The pregnant rats were randomly distributed into 5 groups (A to E) of 6 animals each.

On day 15, rats in group A (negative control) were orally administered with distilled water (2 ml), rats in group B (positive control) were administered with misoprostol (100 µg/kg) and those in groups C to E were treated exactly like those in the control groups, but with varying doses of the extract (125, 250 and 500 mg/kg), respectively.

After three days, the animals were anesthetized, dissected and observed for the presence of fetus within the uterus. Absence of fetus in the uterus indicated occurrence of abortion. The number of rats that aborted per group was recorded and the percentage number of rats that aborted per group calculated:

Percentage number of rats that aborted = (number of rats that aborted/total number of rats per group).

Data analysis

Abortifacient activity of the aqueous extract of *P. dodecandra* leaves was determined by the percentage number of rats that aborted per treated group and the overall activity of the extract compared to that of misoprostol (positive control).

Ethical considerations

Due consideration was accorded to guidelines of Faculty of Medicine Ethical Review Committee (Mbarara University of Science and Technology) and international guidelines concerning animal handling (Institute of Laboratory Animal Resources, Commission on Life Sciences, National Research Council, 1996) during the re-search. The experimental animals were put under deep anesthesia using chloroform before dissection and were euthanized using an overdose of chloroform.

The experiment was carried out with respect to the current guidelines for the care of laboratory animals and ethical guidelines (Institute of Laboratory Animal Resources, Commission on Life Sciences, National Research Council, 1996).

RESULTS

Phytochemical screening

Phytochemical screening of aqueous extract indicated the presence of alkaloids, free amino acids, reducing sugars, steroids, triterpenoids, phenols and tannins (Table 1).

Acute toxicity test results

No deaths were observed. However, at the dose level of 2048 mg/kg, signs of toxicity such as reduced appetite, excessive urination, shivering and sleepiness were observed.

Abortifacient activity

In the effect of aqueous extract of *P. dodecandra* on some abortifacient parameters in pregnant Wistar rats as indicated in Table 2, the highest dose of 500 mg/kg had similar effects to that of the standard drug misoprostol at 100 µg/kg.

DISCUSSION

There is much evidence that shows that some herbal drugs can be used as an abortifacient in rats, mice and even humans (Kazerooni et al., 2006; Koneri et al., 2007). A substance that can disrupt pregnancy could be of interest in human fertility control (Goonasekera et al., 1995). Some of these substances can be used as a contraceptive method specifically as a postcoital contraceptive (Bhargava, 1986; Gandhi et al., 1991).

Preliminary phytochemical screening of the leaf extract of *P. dodecandra* indicated the presence of alkaloids, phenols, steroids, reducing sugars, triterpenoids, tannins and free amino acids. These findings are in agreement with phytochemical screening studies carried out on the plant extract by Ogutu (2012) in Kenya. Of these phytochemicals, alkaloids, phenols and steroids are known to possess abortifacient activity (Yakubu and Bukoye, 2009; Khanna and Chaudhury, 1968).

Table 2. Effect of different doses of aqueous extract of *P. dodecandra* leaves on the abortifacient outcome in pregnant wistar rats.

Parameter	Negative control (distilled water)	Positive control (misoprostol 100 µg/kg)	Doses level of extracts used (mg/kg)		
			125	250	500
Number of rats used	6	6	6	6	6
Number of rats that aborted	0 (0%)	6 (100%)	5 (83.3%)	5 (83.3%)	6 (100%)

The aqueous extract of *P. dodecandra* leaves caused toxic signs such as reduced appetite, sleepiness, excessive urination and shivering at the dose of 2048 mg/kg body weight. Therefore, it is toxic, however, the toxicity is dose dependent. According to the toxicity rating chart, this extract is classified as moderately toxic (0.5 to 5 g) per kilogram body weight in humans (Cassarett and Doull, 2008).

On the basis of the aforementioned results three doses (125, 250, and 500 mg/kg body weight) of *P. dodecandra* were selected for the abortifacient studies.

All the experimental extracts when evaluated for their abortifacient activity, were found to exhibit abortifacient activity. The extract at a dose of 500 mg/kg body weight showed 100% abortifacient activity, doses of 125 and 250 mg/kg showed 83.3% abortifacient activity. In some of the animals vaginal bleeding was observed after 4 h of extract administration. This observation reveals that some of the implants were thus aborted. The absence of fetuses in the uteri of the animals where no vaginal bleeding was observed indicated that the implants may have been aborted when the researchers were not around at night or resorbed by the uterus. This indicated post implantation loss of the fetuses proving the pregnancy terminating potential of *P. dodecandra* leaves.

The overall percentage number of rats that aborted after administration of the extract was 91.7% compared to that of misoprostol which was 100% indicating that the extract of *P. dodecandra* leaves has abortifacient activity. In the present study, exposure of pregnant rats to the aqueous extract of *P. dodecandra* leaves at a dose of 500 mg/kg body weight showed 100% abortifacient activity similar to the effect that was observed with the positive control group in which 100 µg/kg body weight misoprostol was administered, indicating that a dose of 500 mg/kg of *P. dodecandra* is as potent in inducing abortion as misoprostol 100 µg/kg.

Preliminary phytochemical studies indicated the presence of alkaloids, steroids, triterpenoids and phenols in *P. dodecandra* leaf extract. Several of these compounds are known to exhibit antifertility activity (Hiremath and Hanumantharo, 1990; Wang and Ruan, 1996).

Alkaloid-like constituent were reported to be responsible for the suppressant effect on the uterine normal contraction and the high anti-implantation activity exhibited by the aqueous extract of *Graptophyllum pictum* (Stella et al., 2009). Alkaloids (Ergot alkaloids) have been known for more than 2000 years to have adverse effect on pregnancy and can be used alone or in combination with oxytocin to induce abortion (Elderfied, 1980).

Phenolics have also been implicated to be promoters of abortion (Saraiya et al., 1998). In agreement with a study carried out by Yakubu and Bukoye (2009) to determine the abortifacient potentials of the aqueous extract of *Bambusa vulgaris* leaves, the extract contained phenolics and alkaloids which were found to be responsible for its abortifacient activity. Sex hormones being steroidal compounds, the plant sterols were suspected to be responsible for the antifertility effects of the leaves of *P. dodecandra* (Khanna and Chaudhury, 1968). This could therefore mean that alkaloids, phenolic compounds and steroids found in the extract of *P. dodecandra* could be responsible for the abortifacient activity that was found.

Conclusion

The aqueous leaf extract of *P. dodecandra* is moderately toxic; when administered orally, possesses abortifacient activity which could be due to the presence of phenolics, alkaloids and steroids in the extract.

Conflict of interest

The authors declare no conflict of interest.

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REFERENCES

Anees AS, Mohammed A (2004). Practical pharmaceutical chemistry, 1st edn, Faculty of Pharmacy Hamdard University, New Dehli, pp 125-132.
Bhargava SK (1986). Estrogenic and postcoital anticonceptive activity in rats of Butin isolated from *Butea monosperma* seed. J. Ethnopharmacol. 18:95-101
Cassarett L, Doull J (2008). The Basic Science of Poisons, 7th ed, New York, McGraw Hill publishers. p 1454.
Dabhadkar D, Zade V (2013). Abortifacient efficacy of *Indigofera trifoliata* leaves extract on female albino rats. Asian J. Pharm. Clin. Res. 6(suppl. 3):75-79.

Elderfied RC (1980). Biographical memoir for Walter Abraham Jacobs, 51. National Academy of sciences, Washington (DC). pp 247-278.

Gandhi M, Lal R, Sankaranarayanan A, Sharma PL (1991). Post-coital antifertility activity of *Ruta graveolens* in female rats and hamsters. J. Ethnopharmacol. 348:49-59.

Goonasekera MM, Gunawardana VK, Jayasena K, Mohammed SG, Balasubramaniam S (1995). Pregnancy terminating effect of *Jatropha curcas* in rats. J. Ethnopharmacol.47:117-123.

Grimes DA, Benson J, Singh S, Romeo M, Ganatra B, Okonofua FE, Shah IH (2006). Unsafe abortion: The preventable pandemic. Lancet 368:1908-1919.

Hiremath SP, Hanumantharao S (1990). Antifertility efficacy of the plant *Striga lutea* (scrophulariaceae) on rats. Contraception 42:466-477.

Kamatenesi M, Oryem O, Olwa O, Makawiti W (2006). Ethno-pharmacological screening of *Vernonia amygdalina* and Cleome gynandra traditionally used in child birth in Western Uganda, NAPRECA Symposium Book of Proceedings, Madagascar. pp 110-122.

Katende AB, Birnie A, Tengnas B (1995). Useful trees and shrubs for Uganda: Identification, propagation and management for agricultural and live stock communities, Technical Hand book no.10. Regional Soil Conservation Unit. Nairobi, Kenya p 710.

Kazerooni T, Mousavizadeh K, Abdollahee A, Sarkarian M, Sattar A (2006). Abortifacient effect of *Prangos ferulacia* on pregnant rats. Contraception 73(5):554-556

Khanna U, Chaudhury RR (1968). Antifertility screening of plants part I Investigation on *Butea monosperma Linn*. Indian J. Med. Res. 56: 1574-1579.

Koneri R, Saraswayi CD, Balaraman R, Ajeesha E (2007). Antiimplantation activity of the Ethanolic Root extract of Momordicacymbalariafenzl in rats. India J. Pharmacol. (39)2:90-96.

Kress H (2013). Herbal abortives and birth control. Availabe at: http://www.henriettesherbal.com.

Lorke D (1983). A new approach to acute toxicity testing: Arch. Toxicol. 54:275-287.

Lutoti S, Iberet J, Kwiringira W, Kazibwe G (2013). Toxicological review of herbal medicinal products on the Ugandan market. Afr. J. Pharm. Sci. Pharm. 4(1).

Nalule AS, Mbaria JM, Olila D, Kimenju JW (2011). Ethnopharmacology practices in management of livestock helminthes by pastoral communities in the dry lands of Uganda. Livestock Res. Rural Dev. 23(2):36

Ogutu AL, Lilechi DB, Mutai C, Bii C (2012). Phytochemical analysis and antimicrobial activity of *Phytolacca dodecandra, Cucumisacculeatus* and *Erytrinexcelsa*. Int. J. Biol. Chem. Sci. 6(2):692-704.

Prada E, Florence M, Fatima H. A, Rose N, Charles K (2005). Abortion and Post abortion Care In Uganda: A Report from Health Care Professionals and Health Facilities, Occasional report, New York: The Alan Guttmacher Institute. No. 17. Available at: http://www.guttmacher.org/pubs/2005/05/28/or17.pdf

Ritchie HE (2001). The safety of herbal medicine used during pregnancy. Front. Fetal Health 3(10):259-266.

Saraiya M, Berg CJ, Kendrick JS, Strauss LT, Atrash HK, Ahn YW (1998). Cigarette smoking as a risk factor for ectopic pregnancy. Am. J. Obstet. Gynecol. 178(3):493-8.

Selin H (1997). Encyclopedia of the history of science, technology and medicine in non-Western cultures, Dordrecht; Boston: Kluwer Academic.

Stella OOD, Grace EU, Herbert ABC, Samuel AD (2009). Oxytocic and anti-implantation activities of the leaf extract of *Graptophyllum pictum*. Afr. J. Biotechnol. 8:21.

Wang JY, Ruan AI (1996). Triterpenes of *Marsdenia globifera* (for antifertility), Chin. Pharm. J. 31:266-269.

Yakubu MT, Bukoye BB (2009). Abortifacient potentials of the aqueous extract of *Bambusa vulgaris* leaves in pregnantc Dutch rabbits. Contraception 80(3):308-13.

Yeung HW, Feng Z, Li WW, Cheing WK (1987). Abortifacient activity in leaves, roots and seeds of Phytolacca acinosa. J. Ethnopharmacol. 21:31-35.

Zimudzi C (2007). *Adenia lobata* (Jacq.) Engl. In: Schmelzer, G.H. & Gurib-Fakim, A. (ed). Prota 11(1): Medicinal plants/Plantes médicinales 1. [CD-Rom]. PROTA, Wageningen, Netherlands.

Full Length Research Paper

Analysis of rational use of drugs as of facility indicators and patient care indicators practices at four selected hospitals of West Ethiopia: Policy implication

Tadesse Haile Fereja* and Jimma Likisa Lenjesa

Department of Pharmacy, College of Medicine and Health Sciences, Ambo University, Oromia region, Ethiopia.

The international network for rational use of drugs comprises a set of indicators called "drug use indicators" that can be used to assess rational drug use by the patient, health professionals and drug use managements at facility level. These indicators comprise of: Prescribing indicators, patient care indicators and facility indicators. A cross-sectional descriptive study has been conducted to collect the data on patient care and facility indicators, as recommended by the World Health Organization (WHO). Out of 10 public hospitals found in western Oromo, 4 were randomly selected. Data of 160 general ambulatory patients were randomly collected between the months of November, 2012 to March, 2013 from each facility (40 patients per facility). Patient care indicators were measured prospectively by recording consultation time and dispensing time. Percentages of drugs actually dispensed and adequately labeled were determined by examining the drug packages/bottles which the patient had actually received. It was noted whether they had been adequately labeled, viz. whether the name of the patient, the generic name of the drug and when the drug should be taken was written on the label (WHO, 1995). Lastly, the patient's knowledge of when and in what quantity each dispensed drug should be taken was evaluated. In this study, the average consultation time was significantly better in these facilities (18.20 ± 4.3 standard deviation (SD) min) as compared to WHO recommendation which is > 10 min. The dispensing time of drugs in this study was also good (6.56 ± 5.43 SD min) as compared to WHO recommendation which is > 3 min.

Key words: Rational drug use, World Health Organization (WHO) indicators, facility indicators, patient care indicators

INTRODUCTION

Rational use of drugs requires that "patients' receive drugs appropriate to their clinical needs, in doses that meet their own individual requirements, for an adequate period of time and at the lowest cost to them and their community". The concept of rational drug use is old, as evidenced by the statement made by the Alexanderian physician Herophilus 300 B.C that "Drugs are nothing in themselves but are of good if employed with reason and prudence". The concept of rational drug use has been the theme of various national and international gatherings during the past few years. Various recent studies conducted in developed as well as developing countries regarding the safe and effective use of drugs show that irrational drug use is a global phenomenon.

Essential drugs are those that satisfy the health care needs of the majority of the population; they should there-

fore be available at all times, in adequate amounts and in the appropriate dosage forms (Mohammed and Tesfaye, 1997). About one third of the world population lacks access to essential medicines (WHO, Policy Perspective on Medicines, 2002). It is clear that there are differences in public settings regarding the number of health workers and their levels of competence, the particular treatment protocols approved for use by health authorities and those used by agency personnel, the specific compositions of the public populations by age, ethnicity, socio-economic and cultural status and illness profile. However, the effective use of drugs in all public settings depends on rational prescribing, correct dispensing and the treatment adherence by the patient.

In 1993, the World Health Organization (WHO) in collaboration with the International Network for Rational Use of Drugs (INRUD) introduced a set of indicators called "*drug use indicators*" that can be used to assess rational drug use (WHO, 1993). These indicators comprise of: (i) Prescribing indicators that measure the performance of health care providers in several key dimensions related to the appropriate use of drugs. The indicators are based on the prescription practices observed in a sample of clinical encounters taking place at the health institution (Dawit et al., 1998). (ii) Patient care indicators: the time that prescribers and dispensers spend with each patient sets important limits on the potential quality of diagnosis and treatment. Patients for whom drugs are prescribed are expected to receive well-labeled medication and should understand how to take each drug. These indicators measure key aspects of what patients experience at health institutions, and whether they understand how to take their medication appropriately (Negussu, 1991). (iii) Facility indicators: the ability to prescribe or dispense drugs rationally is influenced by many factors of the working environment. Two particularly important components are an adequate supply of essential drugs and access to un-biased information about the drugs. Without these it is difficult for health personnel to function efficiently (Bashrahil, 2010).

Though, many studies had been done on prescription indicators in Ethiopia and other developing countries, there is a lack of such studies on patient care and facility indicators. The present study was aimed at determining the value of these indicators with a view to providing information for further study.

METHODOLOGY

This is a cross-sectional descriptive study which deals with collecting primary data and charts of the current situation concerning the current status of the drug use in the hospitals of West Oromo region and comparing the result with WHO recommendation of patient care and facility indicators. Out of 10 public hospitals found in western Oromo, 4 were systematically selected. The hospitals selected were Ambo Hospital (AH), Gedo Hospital (GH), Nekemte Hospital (NH), and Gimbi hospital (GIH). Data of 160 general ambulatory were randomly collected between the months of November, 2012 to March, 2013 from each facility. The demographic data of each patient including age, sex and prescribed drugs were recorded. Patient care indicators were measured prospectively by recording consultation time and dispensing time. Percentages of drugs actually dispensed and adequately labeled were determined by examining the drug packages/bottles the patient had actually received. It was noted whether they had been adequately labeled, viz. whether the name of the patient, the generic name of the drug and when the drug should be taken was written on them (WHO, 2002). Lastly, the patient's knowledge of when and in what quantity each dispensed drug should be taken was evaluated. Failure to know either of these two points would result in patient's knowledge being scored as inadequate. Data pertaining to the "facility indicators" were gathered at the end of the present study. The prescribers were asked whether any essential drugs list existed in the outpatient department during the study period (WHO, 1993). Fifteen essential drugs formed the checklist to measure the availability of "key drugs", that is drugs that should always be available for the treatment of common health problems, during the study period (WHO, 1993). This information was obtained from the records in the pharmacies. Even if one unit of unexpired product was available, the drug was recorded as being in stock.

The average consultation/dispensing time was calculated by dividing the total time taken (using stop clock) to consult/dispense drugs to series of patients by the number of patients. For the purpose of this study, the time spent on billing and filling the prescription was not considered as part of dispensing time since this time is the time expended by the dispenser independently, excluding the patient, and actually the time used by other person other than the actual dispenser. Percentages of drugs actually dispensed or adequately labeled were computed by dividing the number of drugs dispensed or adequately labeled by the total number of prescribed drugs presented for dispensing. Percentage of patients who had adequate knowledge of the correct dosage schedule was computed by dividing the number of patients who had adequate knowledge of the dosing schedule by the total number of patients interviewed.

RESULTS

Patient care indicators address the key issues of what the patient's experience at health facilities was and how they have been prepared to deal with the pharmaceuticals that have been prescribed and dispensed. According to the data collected, the age composition of the study population revealed that 35 (21.9%) were less or equal to 19 years old, 80 (50.00%) were between the age 20 to 40 years, 45 (28.1%) were for age > 40 years old. From those 160 patients, 63 (39.4%) were females and 97 (60.6%) were males. The marital status of the patients were 63 (39.4%) for females and 97 (60.6%) for males. With regard to educational status of our patients, 53

*Corresponding author. E-mail: tdss_haile@yahoo.com.

Table 1. Distribution of socio-demographic characteristics of the patients (November, 2012 to March, 2013) n=160.

Variables	Frequency	Percent (%)
Educational level		
No formal education	53	33.1
Primary school	39	24.4
High school	45	28.1
Tertiary level	23	14.4
Occupation		
Unemployed	137	85.6
Employed	23	14.4
Gender		
Female	63	39.4
Male	97	60.6
Marital status		
Single	63	39.4
Married	97	60.6
Age category (in year)		
Less or equal to 19	35	21.9
20-40	80	50.0
Greater than 40	45	28.1

(33.1%) of the respondents were illiterate, 39 (24.4%) of respondents were completed primary school, 45 (28.1%) of the respondents were completed secondary level of education and 23 (14.4%) were colleges graduates and above (Table 1).

In this study, the average consultation time was significantly better in these facilities (18.20 ± 4.3 SD min) (Table 2) as compare to values reported by Chedi et al. (2009) from other developing countries (3.4 min). Although it is difficult to estimate optimal time period for a patient encounter, such time is too important to conduct complete patient evaluation and prescribe the therapy for most cases. The dispensing time of drugs in this study was also good (6.56 ± 5.43 SD min) compared to the recommended 2 min (Laing, 1994) but there was medium labeling of drugs (70.6% ± 9.67 SD) as recommended by WHO, that is labeling must be 100%, which is dangerous, especially to illiterate patients as drugs dispensed to them can easily be mixed up at home. The average number of drugs actually dispensed and the average percent of patients with adequate drug dosage knowledge were 2.20 ± 0.6 and 67.5 ± 6 SD, respectively which was also low as of WHO recommendation.

Facility indicators

The National standard treatment guidelines are designed to be used as a guide for treatment choices and as a quick reference in order to help in the overall management of patients. Utmost care has been given by the panel of experts to ensure that the recommendations given in the STG are evidence based. Drugs should only be prescribed when they are necessary, and in all cases the benefit of administering the medicine should be considered in relation to the risks involved. Unadapted prescribing habits lead to ineffective and unsafe treatments, exacerbation or prolongation of the illness, and distress and harm to the patient, as well as a higher cost for the community (Mallet et al., 2001; WHO, 2008). The main drugs prescribed were analgesics, mebendazole and amoxicillin. Other prescriptions included oral rehydration salts (ORS) and others such as antihelminthics, benzylbenzoate emulsions, folic acid, multivitamins and cough mixtures (Table 3).

DISCUSSION

In this study, the average consultation and dispensing time in facilities was 18.20 and 6.56 min. These results transcend those from a study in Niger which were 5.75 and 3.25 min in average (Massele et al., 2001) and in Jordan which were 3.90 min and 28.80 s (Otoom et al., 2002), respectively. Hence, prescribers and dispensers spend more time with their patients. The probable reasons for this variation among health facilities may be due to availability of man power, set up of dispensary area and easy access to essential materials such as drugs and medical equipments. The study result revealed that averagely 28.2% of dispensed drugs were adequately labeled which was less than the study conducted in Islamic republic of Iran where 60% of the drugs were adequately labeled (Cheraghali et al., 2004). This practice should be improved since incomplete labeling of drugs leads to irrational use of medicine. Furthermore the study showed that 82% of patients were able to repeat the correct dosage schedule of the drug they had received; which is relatively low when compared with the WHO recommended value of 100%. The most probable reason for this is the patients educational backgrounds as seen from the patient's socio-demographic study (33% of the study population are illiterate). Other reason that might contribute to this problem may be an overload of patients in the dispensary areas leading to communication problems between dispensers and patients. Almost all of the facilities have their own essential drug list or formulary or standard treatment guideline.

According to the result of this study, the majority of the drugs 776 (76.1%) of the drugs prescribed during the study period in AH, 1142(100%) in GEH, 768(80.7%) in NH and 781(73.5%) in GH were prescribed from the latest edition of Ethiopian essential drug list (EDL) compared with the ideal value of 100% and this is better as compared to the study done by Lifang et al. (2011) that is 64.12%. Percentage of drugs actually dispensed

Table 2. Distribution of patient care indicators of drug use at four hospitals of west Ethiopia, (June, 2013 to March, 2013) n=160.

Indicators	Name of hospitals						P-Value
	AH	GH	NH	GIH	Total	WHO standards	
Consultation time (min) mean (range)	13.35 (4-26)	23.92 (11-31)	17.20 (5-60)	18.15 (5-35)	18.20 (4-60)	10	<0.001*
Dispensing time (min) mean (range)	5.60 (2-10)	12.72 (5-18)	2.72 (1-6)	5.18 (3-9)	6.56 (1-18)	>3	<0.001*
Percent of drug actually dispensed (346/422)	1.70 (0-4)	3.02 (1-6),	2.20 (1-4)	1.72 (0-3)	2.20 (0-6)	100%	<0.001**
Percent of patients with adequate knowledge	70.10 (23)	97.58 (22)	87.13 (27)	69.00 (36)	82% 108(67.5)	100%	0.003**
Percent of drugs with adequate labeling	36	25	16	36	113 (70.6)	100%	<0.001**

*Result of one way ANOVA, **Pearson-chi-square result, AH; Ambo Hospital, GH; Gedo Hospital, GIH; Gimbi Hospital

differed between the pharmacies in the study area (77.2 to 137%). These numbers are slightly lower than the average value obtained in other developing countries (Hogerzeil, 1995). This indicates inappropriate supply of drugs in all the hospitals and therefore a minimum level of health care cannot be guaranteed to all citizens.

Although not a all dispensed drugs were adequately labeled in the four hospitals which was also observed in Cambodia (Chareonkul et al., 2002); figures of 56.2 and 87% have been reported in India (Hazra et al., 2000) and Tanzania (Massele et al., 2001), respectively. When the dispensers were asked about the inadequate labeling, they stated that given their typical workload they hardly got time to interact with the patients and thus they preferred to draw the pictogram and explain how the individual drugs should be taken. The use of pictograms has been shown to improve recall of medical information in people with low literacy skills (Dowse and Ehlers, 2001). However, writing the patient's name and generic name of the drug on the label is necessary (WHO, 1995). This would also help in reducing the risk of dispensing errors (Peterson, 1999). Educational and behavioral intervention and use of pre-packaged drugs would probably improve the dispensing practice. Results that

came up after investigation of the patients' knowledge of correct dosage indicate relatively very high values (70 to 85%). But this does not necessarily reflect reality since the majority of patients were hostile and not willing to repeat the whole dose regimen at the instance of the investigator. Their response "I know the dose" was accepted as positive answer, but it remains doubtful whether they really knew the dose regimens.

One notable characteristic of pharmacies in public health institutions in Kano was their consistency in stocking of essential drugs. This could be the result of the active Drug Revolving Scheme operating in the state and the appropriate policy about the priorities in supplying drugs by its managers. Because of this, the availability of key drugs in the EDL in the four health institutions studied was high (85 to 96%). A similar figure of 86.6% availability of key drugs has been reported from Cambodia (Chareonkul et al., 2002), a lower figure of 54% from Bangladesh (Guyon et al., 1994) and an optimal figure of 100% from Ethiopia (Desta et al., 2002). In spite of the high availability of key drugs in the four health institutions, only AKTH has a hospital formulary. The remaining had neither the formulary nor copies of essential drug list.

Conclusion

The pattern of prescription in terms of polypharmacy was near to optimal which was encourageable. Majority of the drugs were prescribed from Ethiopian EDL, still this is encourageable. The average consultation and dispensing time observed from this study is enough to transfer the necessary information to the clients and improve the adherence. There is a need to improve on patients' knowledge on dispensed drugs and adequate labeling of the dispensed drugs. Still there should be an improvement for stocking of the essential drugs in the facilities. Baseline data gathered by this study can be used by researchers and policymakers to monitor and improve pharmaceutical prescribing and consumption practices in southwest Ethiopia.

Conflicts of interest

The authors declare that they have no conflicts of interest.

REFERENCES

Bashrahil KA (2010). Indicators of rational drug use and health services in Hadramout, Yemen. East Mediterr. Health J.

Table 3. Distribution of facility Indicators at four hospitals of West Ethiopia (June, 2013 to March, 2013) n = 4.

No.	Variables	Names of Hospitals			
		AH	GEH	NH	GH
1	**Latest edition of essential drug list or formulary available**	Yes	Yes	Yes	Yes
2	**Key set of indicator medicines available**				
2.1	Oral Rehydration Solution (ORS)	No	Yes	Yes	Yes
2.2	Cotrimoxazole tablet	Yes	Yes	Yes	No
2.3	Procaine penicillin injection	Yes	Yes	Yes	No
2.4	Amoxicillin	Yes	Yes	Yes	Yes
2.5	Ampicillin	Yes	Yes	Yes	No
2.6	Chloroquine tablet	Yes	Yes	No	Yes
2.7	Coartem	Yes	Yes	Yes	No
2.8	Quinine sulfate	No	No	Yes	Yes
2.9	Ferrous sulfate + folic acid	Yes	Yes	Yes	No
2.10	Mebendazole	Yes	Yes	Yes	Yes
2.11	Tetracycline ointment	Yes	Yes	Yes	No
2.12	Iodine/genitian violet	Yes	Yes	Yes	No
2.13	Benzoic acid + salicylic acid ointment	No	Yes	Yes	No
2.14	Asprine / paracetamol tablet	Yes	Yes	Yes	Yes
2.15	Vitamin A	Yes	Yes	Yes	No
3	**Latest edition of standard treatment guideline (STG) available**	Yes	Yes	Yes	No

AH; Ambo Hospital, GEH; Gedo Hospital, NH; Nekemte Hospital, GH; Gimbi Hospital, Yes; available, No; not available

16(2):151-5.

Chedi BAZ, Abdu-Aguye I, Kwanashie HO (2009). Analysis of patient care and facility indicators in public health institutions in Kano state, Nigeria. Niger. J. Pharm. Sci. 8:2.

Cheraghali AM, Nikfar S, Behmanesh Y, Rahimi V, Habibipour F, Tirdad RA, Bahrami A (2004). Evaluation of availability, accessibility and prescribing pattern of medicines in the Islamic Republic of Iran. Eastern Mediterr. Health J. 10(3):406-415.

Dawit D, Zeleke G, Sheferaw T (1998). A baseline servey on prescribing indicator and factor influencing prescribing in southern Ethiopia. Ethiop. J. Health Dev. 12(2):87-93

Desta Z, Abula T, Ganes A, worku A (2002). Prescribing pattern drugs for outpatient in three hospitals in North West Ethiopia. J. Health Dev. 16(2):183-189.

Hogerzeil HV (1995). Promoting rational prescribing: an international perspective. Br. J. Clin Pharmacol. 39(1):1-6.

Lifang D, Hong Y, Duolao W (2011). Drug prescribing indicators in village health clinics across 10 provinces, of Western China. Fam. Pract. 28:63-67.

Mallet HP, Njikam A, Scouflaire SM (2001). Evaluation of prescription practices and the rational use of medicines in Niger. Sante 11(3):185-96.

Massele AY, Nsimba SE, Rimoy G (2001). Prescribing habits in church-owned primary health care facilities in Dar Es Salaam and other Tanzanian coast regions. East Afr. Med. J. 78(10):510-4.

Mohammed A, Tesfaye S (1997). Pattern of prescription in Jimma Hospital. Ethiop. J. Health Dev.11(3):263-267.

Negussu M (1991). Pattern of drug utilization in Addis Ababa over a seven year interval (1989-1995). Ethop. J. Health Dev.10(1):27-33.

Otoom S, Batieha A, Hadidi H, Hasan M, Al-Saudi K (2002). Evaluation of drug use in Jordan using World Health Organization prescribing indicators. East. Mediterr. Health J. 8(4-5): 537-543.

WHO (1993). How to investigate drug use in health facilities in selected drug use indicator, EDM Research series, No 07. Available at: http://apps.who.int/medicinedocs/en/d/Js2289e/

WHO (2002). Policy Perspective on Medicines; Promoting rational use of medicines; Core components, September, Geneva. Available at: http://apps.who.int/medicinedocs/en/d/Jh3011e/

Full Length Research Paper

Anxiolytic properties of *Melissa officinalis* and associated mechanisms of action: A review of the literature

Bárbara Luisa Fermino[1*], Najeh Nasser Kahlil[1], Juliana Sartori Bonini[1], Romaiana Picada Pereira[2], João Batista Teixeira da Rocha[3] and Weber Claudio Francisco Nunes da Silva[1]

[1]Discente do Curso de Farmácia, Universidade Estadual do Centro-Oeste, UNICENTRO, Guarapuava, PR, Brasil
[2]Departamento de Química, Programa de Pós-Graduação em Química Aplicada, Universidade Estadual de Ponta Grossa, PR, Brasil.
[3]Departamento de Química, Programa de Pós-Graduação em Bioquímica Toxicológica, Universidade Federal de Santa Maria, RS, Brasil.

The anxiety disorders prevalence has significantly increased in society. These disorders can be treated with anxiolytics which, despite great efficacy, may result in several adverse side effects. Several studies have reported that anxiolytic effects result from the indirect action on the GABAergic system and mechanisms related to the cholinergic system. *Melissa officinalis* has been widely utilized for its sedative action and its ability to reduce agitation. Several studies using this plant in different experimental models have demonstrated its low toxicity and lack of side effects. Therefore, this study presents a literature review of the active principles responsible for the anxiolytic effect of *M. officinalis* and the mechanisms involved in this effect.

Key words: *Melissa officinalis*, lemon balm, anxiolytic action.

INTRODUCTION

According to the World Health Organization (WHO), approximately 80% of the world population uses traditional medicine based on empirical knowledge for primary health care (Taiwo, 2007). The use of plants in traditional medicine in Brazil is popular because of the natural diversity observed in the country and low costs the deepening and expansion of studies on herbal medicines have contributed to advances in this usage

(Carvalho, 2011) and discovery of new drugs. *Melissa officinalis* was first described by Carolus Linnaeus in 1753 and initially cited in the French Pharmacopeain. It belongs to the Lamiaceae family and is a perennial lemon-scented herb in the mint family native to the Mediterranean and Southern Europe popularly known as lemon balm (Guginski, 2007). It was introduced to North America and can be found currently in gardens and

roadside fields (Awad et al., 2009). This plant has been widely used because of its several therapeutic actions such as antioxidant (Dastmalchi, 2008; Pereira, 2009; Ribeiro and Bernardo-gil, 2001; Kamdem et al., 2014), anti-inflammatory, hepatoprotective (Birdane, 2007; Bolkent et al., 2005; Encalada et al., 2011), antibacterial, antifungal, antiviral, cholesterol-lowering (Bolkent et al., 2005), antitumor (Saraydin et al., 2012), anti-spas and antidepressant (L´opez et al., 2009).

The plant's sedative properties (Cases, 2011), including a reduction of stress, agitation, and anxiety (Kennedy, 2006) have been widely explored. It is believed that these properties could be related to the citral, which is one of its most abundant secondary metabolites (Lorenzi and Matos, 2002). Thus, as *M. officinalis* becomes a promising alternative to the treatment of anxiety, the understanding of factors that alter the bio-synthesis of citral is critical for a safer use of this herbal medicine.

The prevalence of anxiety disorders has significantly increased in the current society, with 10 million people currently suffering with this pathology (WHO, 2002) in Brazil. Anxiety disorders are usually treated through the use of drugs known as benzodiazepines. Benzodiazepines and barbiturates are the most commonly used despite their significant drawbacks such as physical dependence, tolerance, depression, and interference with memory mechanisms (Taiwo, 2007). Therefore, the search for alternative therapies that are as effective as those in use but with reduced adverse effects is of utmost importance (Baldwin and Ajel, 2007; Kennedy and Scholey, 2004; Millan, 2003; Sinclair and Nutt, 2007; Taiwo, 2007).

Traditional medicines are important options to meet the growing needs of health care; however, there is little scientific evidence ensuring their effectiveness and safety. This study reviewed the information published in the literature about the properties of *M. officinalis*, with the main focus on its anxiolytic roles and the major mechanisms of action involved.

METHODOLOGY

This study conducted an integrative literature review using articles that addressed the effects related to the anxiolytic properties of *M. officinalis* between 1994 and 2014 and indexed in the Scopus, Pubmed, Medline, and SciELO databases and SienceDirect. Eight articles that were published in the last eighteen years and addressed the anxiolytic activity of the plant in experimental models and one in a clinical evaluation were compared in this study.

RESULT AND DISCUSSION

M. officinalis L., Lamiaceae, popularly known as melissa or lemon balm is a perennial herbaceous species

originated in Asia, North Africa, and Southern Europe where it is produced in large scale (Gurčik et al., 2005; Sorensen, 2000). Melissa is reproduced through branch cuttings or imported seeds and plants (Wanderer, 2004). Melissa leaves have been used since ancient times because of its action on the digestive system, mainly due to its carminative and vermifuge properties in the stomach, and as a tonic, antiseptic, and anti-inflammatory (Bertolucci et al., 2008; Sorensen, 2000). Another important use of its leaves and branches is as a condiment (Bertolucci et al., 2008; Carvalho et al., 2005; Couto, 2006).

Citral, citronellal, and geraniol are the main medicinal and condiment constituents in *M. officinalis*. These constituents are found in the essential oil obtained mainly from leaves that can yield between 0.02 and 0.37% of the majority of metabolites (Moradkhanl et al., 2010); the leaves are also used in infusion to produce teas. Hydroxycinnamic acids such as rosmarinic acid, and polyphenols such as tannins and flavonoids, are other constituents of *M. officinalis* reported to play important pharmacological roles (Moradkhanl et al., 2010; Sorensen, 2000).

In vitro studies showed that the essential oil from ethanol extracts of *M. officinalis* leaves contain several metabolites: tannin and rosmarinic derivatives, caffeic acid, flavonoids, and triterpenoid acids. Lorenzi et al. (2002) reported the following compounds as the major components: citronellal (1), citral (2), followed by β-caryophyllene (3), germancrene- D (4), ocimene (5), and citronellol (6) (Lorenzi and Matos 2002) (Figure 1).

Conversely, Allahverdiyev et al. (2004) showed that the most prevalent compounds are β–Cubebene, β–Caryophyllene (the only common compound between the studies), Sesquiterpene alcohol ($C_{15}H_{26}O$), α -Cadinol, Geranial (citral a), and Neral (citral b) (Table 1).

The different results in these two previous studies could come from the use of different extraction and identification methods; Allahverdiyev et al. (2004) used mass spectrophotometry ensuring greater reliability on the results because of the high sensitivity of this method compared to those using older identification methods. However, compounds other than those cited in the present article, such as rosmarinic acid (Boyadzhiev and Dimitrova, 2007), have been found indicating that a wide range of compounds in this plant could hinder the identification of all compounds in its composition. This could be the limiting factor for pharmacological descriptions in other articles found in the literature.

According to Lorenzi and Matos (2002), the anxiolytic action of *M. officinalis* results from the interaction of limonene and citral with GABAA, one of the two ionic channels activated by the ligand responsible for the mediation of γ-aminobutyric acid (GABA), assuming a similar benzodiazepine activity in the plant through nicotinic and muscarinic receptors that are in direct
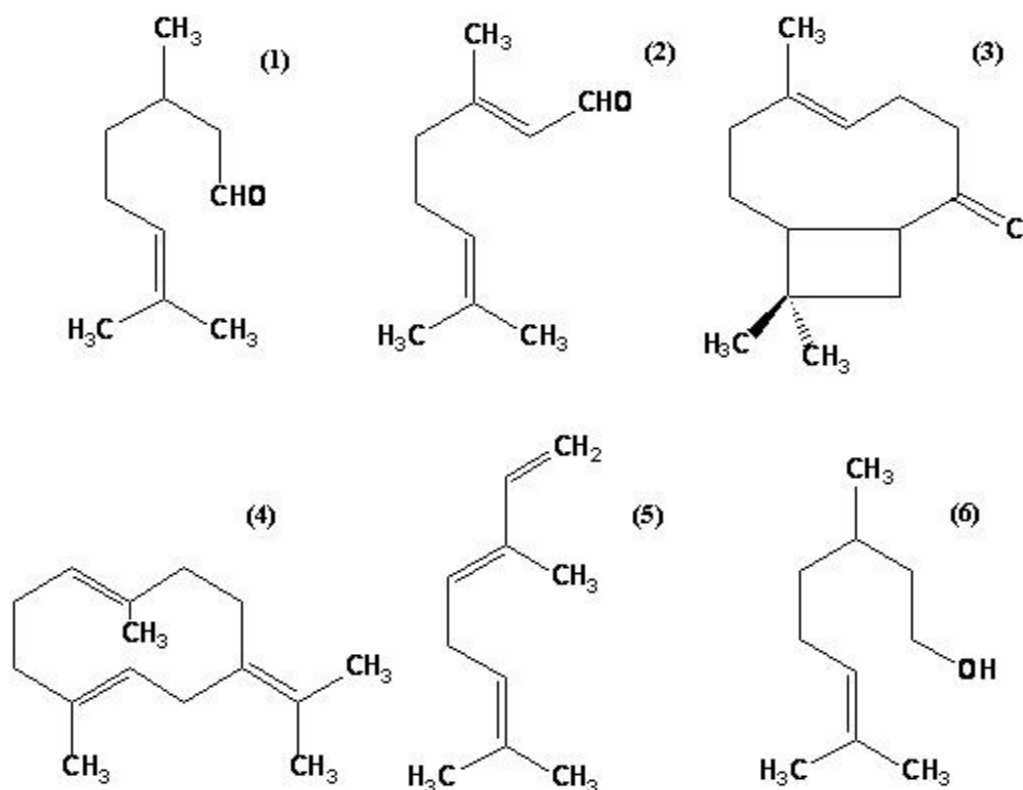
Figure 1. Chemical structure of the major components of *Melissa officinalis* leaves: citronellal (1), citral (2), β-caryophyllene (3), Germancreno-D (4), ocimene (5), and citronellol (6).

connection to the central nervous system (CNS). The study of Wake et al. (2000) working with *M. officinalis* and *Valeriana officinallis* to treat cholinergic receptors showed that the extract from the plant's leaves promoted connections at different levels in the two subtypes of these receptors; the level of connections was higher in the nicotinic subtype because the concentration of this receptor in the human occipital cortex is most expressive.

GABA is an inhibitory neurotransmitter of the central nervous system that reduces nerve impulse transmission between neurons through the hyperpolarization of postsynaptic membranes and the reduction of neuro-transmitter release into the synapse through presynaptic G-protein coupled receptor inhibition of voltage-gated Ca^{++} mechanisms (Weeks, 2009). The GABAergic system is well known as a modulator of cognitive function (Lewis et al., 2008; Menzies et al., 2007) and emotional behavior (Ibarra et al., 2010; Radley et al., 2009; Thoeringer et al., 2009). In this regard, Awad et al. (2009) have reported that rosmarinic acid in plants works by inhibiting the enzyme GABA transaminase (GABA-T), thereby increasing the levels of the neurotransmitter GABA and consequently, reducing anxiety. However, this would only be possible in a moderate stress state, because *M. officinalis* is not efficient when the stress

level is severe (Ibarra et al., 2010). The GABAA receptors are ionic channels that mediate the effects of GABA, producing an inhibitory action through the opening of chloride channels preventing a neuronal action potential. This is seen as the mechanism of action of diazepam and is regarded as one of the possible mechanisms of action of *M. officinalis* (Abuhamdah et al., 2008; Akhondzadeh et al., 2003; Kennedy et al., 2002; Wecker and Catalano, 2006).

Wake et al. (2000) reported anxiolytic effects of *M. officinalis* on the CNS besides the proposed mechanism of specific metabolites connections in the herb, such as limonene and citral on the GABAA neurotransmitter. These effects occur through cholinergic receptors, where muscarinic receptors produce antagonistic effects especially on the M1 receptor. This receptor is located in the nerve ganglia and front-parietal cortex and acts by mediating excitatory postsynaptic potential due to stimulation of intracellular calcium entry (Gerber et al., 2001; López et al., 2009). Wake et al. (2000) analyzed Wistar rats in traditional behavioral models such as the Y-maze, social interaction test, forced swimming, and elevated cross maze and used tea from the leaves of *M. officinalis* as the testing sample. These authors demonstrated anxiolytic effects on the CNS through

Table 1. Percentage composition of identified compounds in *M. officinalis* total oil (Allahverdiyev et al. 2004).

Compound	%
β-Cubebene	15.41
β-Caryophyllene	14.24
Sesquiterpene alcohol ($C_{15}H_{26}O$)	7.39
α-Cadinol	7.18
Geranial (citral a)	6.62
Neral (citral b)	5.82
Cadinol isomer	3.96
trans-b-ocimene	3.96
β-Cadinene	3.62
Citronellal	2.92
β-Cedrene	2.53
α-Bisabolene	2.51
Nerolidol	2.36
Nonanal	2.34
α-Copaene	2.26
Calarene	2.12
γ-Elemene	1.7
Pinocamphone	1.34
Linalool	1.32
α-Cubebene	1.27
β-Elemene	0.89
1-Hepten-3-ol	0.5
6-Metyl- 5- heptene-2-one	0.42
Geraniol	0.38
cis-β-ocimene	0.37
Identified	93.43
Unidentified	6.57

the ingestion of high doses of plant extracts and teas, which did not induce respiratory depression or depressive attenuation at the level of the CNS frame. However, Coimbra (1994) pointed out that the treatment with *M. officinalis* essential oil in high doses can lead to mutagenic and neurotoxic effects.

Kennedy et al. (2002) used several concentrations of *Melissa's* essential oils (0.6, 1.2, and 1.8 g/kg/day) in humans to evaluate the anxiolytic action of *M. officinalis*, which occurs in the CNS by modulation of mood and cognitive processes. These authors reported that the most effective anxiolytic action was observed with the dose of 1.8 g/kg/day because it acted on the cholinergic system by decreasing stress and agitation in patients, thereby, confirming the proposed anxiolytic effect after ingestion of the plant's extract.

Wake et al. (2000) indicate that the main mechanism of action of *M. officinalis* extracts is based on its interaction with cholinergic receptors, the acetylcholine receptor (ACh). It is pointed out that *Melissa* will act displacing the molecule [3H] - (N)-nicotinic nicotinic, [3H] - (N)-escopolaminica, and muscarinic receptors by increasing ACh released after nerve stimulation in a mechanism that may be involved in improving cognitive function and reducing agitation. This hypothesis is not confirmed, and details of such a mechanism are still unknown.

Nevertheless, in contrast to Kennedy et al. (2002), Abascal and Yarnell (2004) reported in a randomized, double-blind trial control study using placebo with 20 healthy volunteers, that *M. officinalis* promoted an improvement in attention and stress reduction with a reduced dose (300 mg/kg/day) and decreased alertness and memory loss with an increased dose (900 mg/kg/day). However, the study failed to confirm significant cholinergic action.

Akhondzadeh et al. (2003) administered *M. officinalis* leaf extracts (600 mg/kg/day) to a group of Alzheimer's disease patients at mild to moderate stages of the disease. The study demonstrated that this treatment during 16 weeks resulted in a significant cognitive

improvement and reduction in the agitation experienced by some patients with this disease. This study demonstrates a possible effective treatment of Alzheimer's disease with *M. officinalis* resulting from modulatory actions on mood and cognitive performance, and on acetylcholine receptors in the CNS, following acute administration.

Nowadays, *M. officinalis* is not individually used as a pharmacological treatment for any disease. It is widely used in conjunction with another plant such as in Sonhare® whose pharmacogens from *Valeriana officinalis L.* and *M. officinalis L.* have therapeutic indications in relieving sleep difficulties, tension, restlessness, and irritability. The Sonhare® has a medication package and insert information sheet that do not inform which part of the plant is used or illustrate self-medication usage. Nevertheless, this is a controlled medication that is an excellent option in the prophilaxis of advanced stress (Moura, 2006).

There are few in-depth studies available reporting on the action in other systems on this subject because they failed to address pharmacological aspects. However, some can be cited such as the digestive system (Simmen et al., 2006; Schemann et al., 2006) in which the action can be linked to gastrointestinal motility reduction (Bolkent et al., 2005). Moreover, Sadraei et al. (2003) reported anti-spasmodic effects in the ileum due to one of its major components, indicating that *M. Officinalis* represents a good choice of herbal treatment for spastic episodes in the gastrointestinal system.

This plant can present a protective action in the hepatic system because of the presence of phenolic compounds (Simmen et al., 2006; Schemann et al., 2006). However, a study conducted by Müzell (2006) pointed that *M. officinalis* displayed hepatotoxic effects after toxicity was induced in mice by Acetaminophen, resulting in inhibition or modulation of the activity of cytochromes P450 conferred by flavonoids present in the plant, and enabling these to increase or reduce the concentrations of various therapeutic drugs in plasma (Hodek et al., 2002). In the same study, Müzell (2006) covered the renal system, seeking the possibility of its protection from the plant's activity; however, the results were unsatisfactory because the drug toxicity was intensified in this case.

Anti-inflammatory activity is also promoted by phenolic compounds present in the plant, such as flavonoids, that have the ability to inhibit the activity of monooxygenases, lipooxigenases, cyclooxygenases (Svobodová et al., 2003), oxidoredutases, and hydrolases such as the hyaluronate lyase that catalyzes the degradation of hyaluronic acid; inhibitions can be competitive in some cases or allosteric in others (Havsteen, 2002). The rosmarinic acid is among the variety of compounds cited or not by different authors, which has shown anti-inflammatory activity and inhibitory activity to 5-lipoxigense, 3R-hydroxysteroid dehydrogenase, and lipid peroxidation as reported by Nakazawa et al. (1998). This compound features astringent, antioxidant, and anti-inflammatory activity by inhibiting lipooxigenases and cyclooxygenases, antibacterial and antiviral activity, and antimutagenic effect (Pereira et al., 2005; Petersen and Simmonds, 2003).

In addition to the anti-inflammatory activity, the antiviral activity to *Herpex simplex* can be cited, which was reported for the first time by May and Willuhn (1978). In this context, Schnitzler et al. (2008) observed reduced viral replication activity when using *M. officinalis* oil *in vitro*.

Conclusion

The use of traditional medicine presents a significant risk to public health because of the lack of knowledge regarding drug interactions and possible toxic effects. Research studies assessing the clinical efficacy of plants are necessary. Based on this literature review, it was concluded that extracts of *M. officinalis* have effective anxiolytic activity in reducing stress and physiological disturbances due to its direct interaction with the CNS and the cholinergic and GABAergic systems. Its mechanism of action is still controversial. Some authors suggest it is active on the GABAergic system and others on the cholinergic system. Abascal and Yarnell (2004) report no significant action on the cholinergic system while Wake et al. (2000) indicate that the main mechanism of action of *M. officinalis* is based on its interaction with cholinergic receptors.

Conflict of interest

Authors declare that there are no conflicts of interest

REFERENCES

Abuhamdah S, Liping Huang, MSJE, Howes MJR, Ballard C, Holmes C, Burns, A, Perry EK, Lees PTFG, Chazot PL (2008). Pharmacological profile of an essential oil derived from *Melissa officinalis* with anti-agitation properties: focus on ligand-gated channels. J. Pharm. Pharmacol. 60:377-384.

Akhondzadeh S, Noroozian M, Mohammadi M, Ohadinia S, Jamshidi A, Khani M (2003). Melissa officinalis extract in the treatment of patients with mild to moderate Alzheimer's disease: a double blind, randomised, placebo controlled trial. J. Neurol. Neurosurg. Psychiatry 74:863-866.

Allahverdiyev A, Duran N, Ozguven M, Koltas S (2004). Antiviral activity of the volatile oils of *Melissa officinalis*. Against Herpes simplex virus type-2. Phytomedicine 11:657-661.

Awad R, Muhammad A, Durst T, Trudeau VL, Arnason JT (2009). Bioassay-guided Fractionation of Lemon Balm (*Melissa officinalis* L.) using an *in vitro* measure of GABA transaminase activity. Phytother. Res. 23(8):1075-1081.

Baldwin DS, Ajel K (2007). Development and evaluation of psychotropic drugs. Psychiatry 6:279-283.

Bertolucci S, Lameira A, Pinto J (2008). Guia das plantas medicinais. In: Lameira, A. O.; Pinto, J. E. B. P., in: Belem (Ed.), Plantas medicinais: do cultivo, manipulacao e uso a recomendacao popular pp. 199-201.

Birdane YEA (2007). Anti-inflamatory and nociceptive effects of *Melissa officinalis* L. in rodents. Rev. Med. Vet.158:75-81.

Bolkent S, Yanardag R, Karabulut-Bulan O, Yesilyaprak B (2005). Protective role of Melissa Officinalis L. extract on liver os hyperlipidemic rats: A morphological and biochemical study. J. Ethnopharmacol. 99:391-398.

Carvalho H, Cruz, F, Wiest, J (2005). Atividade antibacteriana em plantas com indicativo etnografico condimentar em Porto Alegre, RS/Brasil. Revista Brasileira de Plantas Medicinais Botucatu 7:25-32.

Carvalho NEA (2011). Evaluation of the genotoxic and antigenotoxic potential of Melissa officinalisinmice. Genet. Mol. Biol. 34:290-297.

Cases J, Ibarra A, Feuillère N, Roller M, Sukkar SG (2011). Pilot trial of Melissa officinalis L. leaf extract in the treatment of volunteers suffering from mild-to-moderate anxiety disorders and sleep disturbances. Med. J. Nutr. Metab. 4:211-218.

Coimbra R (1994). Manual de Fitoterapia. 2nd edn. Editora Cejup, Belem, Brazil.

Couto MEO (2006). Coleção de plantas medicinais, aromáticas e condimentares, Embrapa Clima Temperado. Documentos p 157.

Dastmalchia K, Damien Dormana HJ, Oinonena PP, Darwisd Y, Laaksoa I, Hiltunena R (2008). Chemical composition and *in vitro* antioxidative activity of a lemon balm (*Melissa officinalis* L.) extract. Food Sci. Technol. 431:391-400.

Encalada MA, Hoyos KM, Rehecho S, Berasategi I, de Ciriano MG, Ansorena D, Astiasarán I, Navarro-Blasco I, Cavero RY, Calvo MI (2011). Anti-proliferative effect of Melissa officinalis on human colon cancer cell line. Plant Foods Hum. Nutr. 66:328-334.

Gerber DJ, Sotnikova TD, Gainetdinov RR, Huang YS, Caron MG, Susumu Tonegawa S (2001). Hiperactivity, elevated dopaminergic transmission, and response to amphetamine in M1 muscarinic acetylcholine receptor deficient. Neurobiol. 98:15312-15317.

Guginski G (2007). Análise da atividade farmacológica do extrato etanólico obtido da Melissa officinalis. *Programa de pós-graduação em farmacologia* Universidade federal de santa catarina.

Gurčik Ľ, Dubravska R, Miklovičova J (2005). Economics of the cultivation of *Salvia officinalis* and *Melissa officinalis*. Agricural Economics, West Lafayette 51:348-356.

Havsteen BH (2002). The biochemistry and medical significance of the flavonoids. Pharmacol. Ther. 96:67-202.

Hodek P, Trefil P, Stiborova M (2002). Flavonoids-potent and versatile biologically active compounds interacting with cytocromes P450. Chem. Biol. Interact.139:1-21.

Ibarra A, Feuillere N, Roller M, Lesburgere E, Beracochea D (2010). Effects of chronic administration of *Melissa officinalis* L. extract on anxiety-like reactivity and on circadian and exploratory activities in mice. Phytomedicine 17:397-403.

Kamdem JP, Adeniran A, Boligon AA, Klimaczewski CV, Elekofehinti OO, Hassan W, Ibrahim M, Waczuk EP, Meinerz DF, Athayde ML (2013). Antioxidant activity, genotoxicity and cytotoxicity evaluation of lemon balm (*Melissa officinalis* L.) ethanolic extract: Its potential role in neuroprotection. Ind. Crops Prod.51:26-34

Kennedy D (2006). Anxiolytic effects of a combination of *Melissa officinalis* and *Valeriana officinalis* during laboratory induced stress. Phytother. Res. 20:96-102.

Kennedy DW, Scholey AB (2004). Attenuation of laboratory-induced stress in humans after acute administration of Melissa officinalis (Lemon Balm). Psychosom. Med. 66:607-613.

Kennedy DO, Scholey AB, Tildesley NT, Perry EK, Wesnes KA (2002). Modulation of mood and cognitive performance following acute administration of single doses of Melissa officinalis (Lemon Balm). Pharmacol. Biochem. Behav.72:953-964.

Lewis DA, Cho RY, Carter CS, Eklund K, Forster S, Kelly MA, Montrose D (2008). Subunit selective modulation of GABA type A receptor neurotransmission and cognition in schizophrenia. Am. J. Psychiatry 165:1585-1593.

López VC, MartıS, Go´mez-Serranillos MP, Carretero, ME Ja¨ger, AK,

Calvo MI (2009). Neuroprotective and Neurological Properties of *Melissa officinalis*. Neurochem. Res. 34.

Lorenzi H, Matos F (2002). Plantas medicinais no Brasil: nativas e exóticas.

May G, Willuhn G (1978). Antiviral effect of aqueous plant extracts in tissue culture [in German; English abstract]. Arzneimittelforschung 28:1-7.

Menzies L, Ooi C, Kamath S, Suckling J, Mc Kenna P, Fletcher P, Bullmore E, Stephenson C (2007). Effects of gamma-aminobutyric acid modulating drugs on working memory and brain fucntion in patients with schizophrenia. Arch. Gen. Psychiatry 64:156-167.

Millan M (2003). The neurobiology and control of anxious states. Prog. Neurobiol.70:83-244.

Moradkhani H, Sargsyan E, Bibak H, Naseri B, Sadat-Hosseini M, Fayazi-Barjin A, Meftahizade H (2010). Melissa officinalis L., a valuable medicine plant: A review. J. Med. Plants Res. 4:2753-2759.

Moura LC (2006). Fitoterapia aplicada em Farmácias: Estudo dos Fitoterápicos Ansiolíticos, Sedativos e Antidepressivos, Farmácia. Escola de Saúde Pública do Ceará., Especialização em assistência farmacêutica.

Müzell DP (2006). Preopriedades Biológicas de Extratos de *Melissa officinalis* L. (Lamiaceae) em Ratos Wistar, Biociências. PUC-RS. Especialização em Biologia Molecular.

Nakazawa T, Ohsawa K (1998). Metabolism of Rosmarinic Acid in Rats. J. Nat. Prod. 61:993-996.

Pereira RP, Fachinetto R, de Souza Prestes A, Puntel RL, Santos da Silva GN, Heinzmann BM, Boschetti TK, Athayde ML, Bürger ME, Morel AF, Morsch VM, Rocha JB (2009). Antioxidant effects of different extracts from Melissa officinalis, Matricariarecutita and Cymbopogoncitratus. Neurochem. Res.34:973-983.

Pereira P, Tysca D, Oliveira P, Brum LFS, Picada JN, Ardenghi (2005). Neurobehavioral and genotoxic aspects of rosmarinic acid. Pharmacol. Res. 52:199-203.

Petersen M, Simmonds MSJ (2003). Rosmarinic acid. Phytochemistry 62:121-25.

Radley JJ, Gosselin KL, Sawchenko PE (2009). A discrete GABAergic-relay mediates medial prefrontal cortical inhibition of the neuroendocrine stress response. J. Neurosci. 29:7330-7340.

Ribeiro M, Bernardo-gil MG (2001). *Melissa officinalis*, L.: study of antioxidant activity in supercritical residues. J. Supercrit. Fluids 21:51-60.

Saraydin SU, Tuncer E, Tepe B, Karadayi S, Özer H, Şen M, Karadayi K, Inan D, Elagöz Ş, Polat Z, Duman M, Turan M (2012). Antitumoral effects of Melissa officinalis on breast cancer *in vitro* and *in vivo*. Asian Pac. J. Cancer Prev.13:2765-2770.

Schemann M, Michel K, Zeller F, Hohenester B, Rühl A (2006). Region-specific effects of STW 5 (Iberogast) and its components in gastric fundus, corpus and antrum. Phytomedicine 13:90-99.

Schnitzler P, Schuhmacher A, Astani A, Reichling J (2008). *Melissa officinalis* oil affects infectivity of enveloped herpes viruses. Phytomedicine 15 :734-740.

Simmen U, Kelber O, Okpaneji SN, Jaeggi R, Buetler B, Weiser D (2006). Binding of STW 5 (Iberogast) and its components to intestinal 5-HT, muscarinic M3 and opioid receptors. Phytomedicine 13:51-55.

Sinclair L, Nutt D (2007). Anxiolytics. Psychiatry 6:284-288.

Sorensen J (2000). *Melissa officinalis* – essential oil – authenticity, production and pharmacological activity – a review. Int. J. Aromather.10:7-15.

Svobodová A, Psotová J, Walterová D (2003). Natural phenolics in the prevention of UV-induced skin damage. A review. Biomed Pap Med Fac Univ Palacky Olomouc Czech Repub. 147(2):137-145.

Taiwo A (2007). Alterações Comportamentais decorrentes da administração de Mellissa officinali sem ratos, Faculdade de Ciência da Saúde. Universidade de Brasília.

Thoeringer C, Erhardt A, Sillaber I, Mueller M, Ohl F, Holsboer F, Keck M (2009). Long-term anxiolytic and antidepressant-like behavioural effects of tiagabine, a selective GABA transporter-1 (GAT-1) inhibitor, coincide with a decrease in HPA system activity in C57BL/6mice. J. Psychopharmacol. 24:733-743.

Wake G, Court J, Pickering A, Lewis R, Wilkins R, Perry E (2000). CNS

acetylcholine receptor activity in European medicinal plants traditionally used to improve failing memory. J. Ethnopharmacol. 69:105-114.

Wanderer M (2004). Produção de mudas e rendimento de biomassa de melissa (*Melissa officinalis* L.) sob diferentes espaçamentos de plantas e cobertura de solos., Mestrado em Fitotecnia. Universidade Federal do Rio Grande do Sul. p 122.

Wecker L, Catalano G (2006). Tratamento da ansiedade e dos distúrbios do sono: In: Brody Farmacologia Humana. Elsevier 285-286.

Weeks BS (2009). Formulations of dietary supplements and herbal extracts for relaxation and anxiolytic action. Relarian. Med. Sci. Monit. 15:256-262.

WHO (2002). Monographs on selected medicinal plants. WHO Graphics 2, 351.

Full Length Research Paper

Effect of *Citrus paradisi* and *Citrus sinensis* on glycemic control in rats

Neelam Mallick and Rafeeq Alam Khan*

Department of Pharmacology, Faculty of Pharmacy, University of Karachi, Karachi 75270-Pakistan.

This study was conducted to explore the effects of *Citrus sinensis* (orange juice) and *Citrus paradisi* (grapefruit juice) at three different doses alone and their two combinations on plasma insulin and blood glucose levels in healthy and diabetic rats. Diabetes was induced by alloxan after which rats were treated with *C. sinensis* and *C. paradisi* juices for six weeks, blood glucose and plasma insulin concentration was estimated. *C. sinensis* showed significant reduction in blood glucose and a significant rise in plasma insulin at all three doses. However *C. paradisi* revealed highly significant fall in blood glucose and highly significant rise in plasma insulin levels only at 0.5 ml/kg. Whereas combination dose group CSP-2 (5 + 0.3 ml/kg) showed highly significant reduction in blood glucose and highly significant rise in plasma insulin levels as compared to diabetic control. These results suggest that flavonoids and other essential nutrients present in citrus fruits juices might be responsible for these effects. Hence, it may be concluded that these juices may be used in combination to produce a synergistic effect in decreasing blood glucose and elevating plasma insulin levels.

Key words: Grape fruit, orange, glucose level, insulin.

INTRODUCTION

Diabetes mellitus is a metabolic disorder resulting in severe immediate and continuing consequences extending from brain damage to heart disease (American Diabetes Association, 2009). According to the report of International Diabetes Federation, the estimated number of people with diabetes increased universally in recent years and is expected to reach 380 million in 2025 (Samreen, 2009). Large number of agents is available for treatment but is associated with various serious adverse effects (Tahrani et al., 2010; Patel et al., 2012). Plants have been used over a long time for the treatment of different ailments. Fruits and vegetables have gained

importance for the management of diabetes mellitus, for example pomegranate, grapes, guava, lemon, tomatoes and grape fruits. They have the capacity to re-establish function of pancreatic tissues by increasing insulin output, inhibiting intestinal absorption of glucose or helping metabolites in insulin dependent processes (Javascript, 2002; Free encyclopedia, 2002; Punitha et al., 2006; Malviya et al., 2010; Riaz et al., 2013).

This study was conducted on fresh juices of two fruits, *Citrus sinensis* (orange) and *Citrus paradisi* (grapefruit) belonging to Rutaceae family, since plants and plant derived phytochemical have great potential to treat and

control diabetes (Behera and Yadev, 2013). Grapefruit contains phytochemicals including limonoids and lycopene. It is also an exceptional source of vitamin C, dietary fiber, vitamin A, potassium, folate and vitamin B5 (Mateljan, 2006). It also contains high levels of iron, calcium and other minerals. Pink and red varieties of grapefruits are rich in beta carotene, high in fiber and low in calories. They possess protective plant chemicals like phenolic acid, limonoids, terpenes, monoterpenes and bioflavonoids which protect against cancer and heart disease. The major bioflavonoid in grapefruit is naringin that gives grapefruit juice bitter taste (Giovannucci et al., 2002; Armando et al., 1998). Naringin exerts diverse pharmacological effects like antioxidant activity, blood lipid-lowering effect (Gorinstein et al., 2006), anti-carcinogenic activity (Armando et al., 1998) and inhibition of selected cytochrome P450 enzymes including CYP3A4 and CYP1A2 (Gao et al., 2006). Naringin being one of the components of these fruits have been reported for its antidiabetic effects (Pari and Suman, 2010). Grapefruit enhance appetite and is employed for its digestive, stomachic, antiseptic and diuretic properties (Herbal medicine, 2000).

Orange being a rich source of nutrients has also been known for a number of health and nutritional benefits. Orange contains nearly two hundred phytonutrients and flavonoids, which have shown activity against different types of cancers. They also have been reported to have strong anti-inflammatory and anti-oxidant properties and prevent bone loss (Liu et al., 2012; Peluso, 2006; Gao et al., 2006; Chiba et al., 2003). The essential oils in orange juice (*C. sinensis*) contain many constituents, including monoterpenes and sesquiterpenes with d-limonene as a major constituent (Graciela et al., 2003). Orange juice has been reported for cholesterol lowering effect in animal models (Kurowska et al., 2000) as well as humans (Roza et al., 2007). Its anti-inflammatory role in different disease is also well documented (Buscemi et al., 2012; Ghanim et al., 2010). Narirutin or Naringenin 7-O-rutinoside is another important flavonoid present abundantly in orange juice (Sawalha et al., 2009). It is absorbed well and shows good bioavailability (Manach et al., 2003). It is also shown to possess anti-inflammatory (Ha et al., 2012; Funaguchi et al., 2007), anti-allergic and anti-asthmatic effects (Rogerio et al., 2010; Funaguchi et al., 2007). Orange and grape fruit are both rich in phytochemical, flavonoids and vitamins which revealed to have strong anti-inflammatory and antioxidant properties (Liu et al., 2012; Peluso, 2006; Gao et al., 2006; Chiba et al., 2003).

MATERIALS AND METHODS

Animals

Present study was conducted after approval of the proposal by board of advance studies and research, University of Karachi on adult Wister rats with mean body weight of 220 ± 10 g. Animals were

kept under controlled condition of temperature 23 ± 2°C and humidity 50 to 60%, with free access to food and water. Five rats were housed in each plastic cage measuring 32" × 18" × 16".

Citrus sinensis

C. sinensis was obtained from local market and recognized by Center of Plant Conservation, University of Karachi. The voucher specimen no.CS-10-10 was placed in the Department of Pharmacognosy, University of Karachi. *C. sinensis* was peeled and pressed by hand to yield fresh juice which was then used immediately after filtration. *C. sinensis* juice was administered in three different doses that is, 2, 5 and 8 ml/kg according to body weight through oral route.

Citrus paradisi

C. paradisi was also bought from local market, recognized by Center of Plant Conservation, University of Karachi. The voucher specimen no. CP-09-10 was kept in the Department of Pharmacognosy, University of Karachi. The fruits were peeled, pressed and fresh juice so obtained was filtered and used soon after yielding. *C. paradisi* was given in three doses that is, 0.1, 0.3 and 0.5 ml/kg according to body weight through oral route.

Combinations of *C. sinensis* and *C. paradisi*

C. sinensis and *C. paradisi* were also given orally in two combined doses that is, 2 + 0.1 and 5 + 0.3 ml/kg, and were abbreviated as CSP-1 and CSP-2.

Induction of diabetes

Diabetes was induced in overnight fasted rats by means of a single dose of alloxan monohydrate (Sigma chemicals, USA). Alloxan was administered in the dose of 180 mg/kg in normal saline by intraperitoneal injection. The glucose level in plasma was determined at 72 h after the administration of alloxan (Rohilla and Ali, 2012). Animals with blood glucose concentration more than 250 mg/kg were considered diabetic and used for the study.

Design of experiment

All rats were distributed in eleven groups each comprising of ten animals. One group received saline and served as normal control, while remaining ten groups induced diabetes received *C. sinensis* (three groups), *C. paradisi* (three groups), while two groups received *C. sinensis* and *C. paradisi* in combination, one group received standard drug glibenclamide and one group received saline served as diabetic control. All doses were given by gastric intubation on once daily basis for a period of six weeks. Entire study was performed under NACLAR guideline (Bernard, 2004). At the end of the investigational period, animals were deprived of food overnight, sacrificed by amputation and blood samples were collected in gel tubes and 3.2% sodium citrate containing tubes (9:1 v/v).

Estimation of blood glucose

Blood glucose was estimated by two methods, first through Accu-Chek glucometer by taking blood from tail vein of rats, after 72 h of intraperitoneal injection of alloxan to check the induction of diabetes. Second through blood collected in gel tubes. Serum was

Table 1. Effect of *C. sinensis* on glucose and plasma insulin levels.

Parameters	Groups				
	Normal control	Diabetic control	*C. sinensis* 2 ml/kg	*C. sinensis* 5 ml/kg	*C. sinensis* 8 ml/kg
Blood glucose (mg/dl)	93.7 ± 4.41	292.5 ± 19.53	227.81 ± 17.29*	218.65 ± 24.83*	223.9 ± 19.70*
Plasma insulin (µU/ml)	15.6 ± 0.35	7.9 ± 1.01	10.08 ± 0.35*	9.93 ± 0.47*	10.28 ± 9.1*

n=10, Values are means ± S.E.M. *P ≤ 0.05 significantly different as compared to control.

Table 2. Effect of C. paradisi on glucose level and plasma insulin levels.

Parameters	Groups				
	Normal control	Diabetic control	*C. paradisi* 0.1 ml/kg	*C. paradisi* 0.3 ml/kg	*C. paradisi* 0.5 ml/kg
Blood glucose (mg/dl)	93.7 ± 4.41	292.5 ± 19.53	186.39 ± 23.47**	180.37 ± 20.03**	169.93 ± 22.55**
Plasma insulin (µU/ml)	15.6 ± 0.35	7.9 ± 1.01	8.7 ± 0.68	10.29 ± 0.56*	11.01 ± 0.23**

n=10, Values are means ± S.E.M. *P ≤ 0.05 significantly different as compared to diabetic control. **p≤ 0.005 highly significant as compared to diabetic control

Table 3. Effect of combination doses of *C. sinensis* - *C. paradisi* and glibenclamide on glucose and plasma insulin levels.

Parameters	Groups				
	Normal Control	Diabetic Control	CSP -1 Treated	CSP -2 Treated	Glibenclamide
Blood glucose (mg/dl)	93.7 ± 4.41	292.5 ± 19.53	221.38 ± 14.5*	179.3 ± 27.06**	158.01 ± 18.37**
Plasma insulin (µU/ml)	15.6 ± 0.35	7.9 ± 1.09	8.76 ± 0.62	11.65 ± 0.54**	14.03 ± 0.62**

n=10, Values are means ± S.E.M. *P ≤ 0.05 significantly different as compared to control. **p≤ 0.005 highly significant as compared to diabetic control. CSP -1: 2+0.1 ml/kg/day *C. sinensis* and *C. paradise*, respectively. CSP -2: 5+0.3 ml/kg/day *C. sinensis* and *C. paradise*, respectively. Glibenclamide: 2.5 mg/kg.

separated by Humax 14K centrifuge at 2000 rpm for 10 min and then commercial kit of glucose (Human Diagnostic, Germany) was used to estimate blood glucose by Humalyzer 3000 Human Germany.

Estimation of plasma insulin

Blood was collected in 3.2% sodium citrate tubes and plasma was parted by Humax 14 K centrifuge at 3000 rpm for 15 min. Insulin was estimated using commercial ELISA insulin kit (Accu-Bind, Elisa Microwells, USA).

Statistical analysis

Entry of data and analysis was performed using 17th version of superior performance statistical software (SPSS). Quantities were presented as mean ± SD with 95% confidence interval. Analysis of variance (ANOVA) followed by post hoc was performed for comparisons of values with control. Values of p < 0.05 were considered statistically significant and p < 0.005 highly significant.

RESULTS

Table 1 reveals the effect of *C. sinensis* on blood glucose and plasma insulin levels in diabetic and control rats. There was significant decrease in blood glucose at 2, 5 and 8 ml/kg of *C. sinensis* in a dose dependent manner than diabetic control, while there was significant rise in insulin level at all three dose as compared to diabetic control. Table 2 displays the effect of *C. paradisi* on blood glucose and plasma insulin levels in diabetic and control animals. There was highly significant decrease in blood glucose at 0.1, 0.3 and 0.5 ml/kg of *C. paradisi* in a dose dependent manner than diabetic control. While there was highly significant increase in plasma insulin levels at 0.5 ml/kg and significant increase at 0.3 ml/kg. However, there was no change in plasma insulin level at 0.1 ml/kg. Table 3 shows the comparative effect of combination doses of *C. sinensis* and *C. paradisi* with standard drug glibenclamide on plasma glucose and insulin levels in diabetic control and normal control animals. There was highly significant decrease in blood glucose in animals treated by CSP-2 combination and glibenclamide. While significant decrease was observed in animals treated with CSP-1 combination as compared to diabetic control. There was also highly significant rise in plasma insulin at CSP-2 and glibenclamide treated animals as compared

to diabetic control. However, there was no change in plasma insulin level at CSP -1.

DISCUSSION

Type-I diabetes is categorized by a loss of insulin-producing beta cells islets of Langerhans in the pancreas leading to decrease in insulin. Diabetes was induced by the administration of alloxan to the experimental animals causing destruction of beta cells, a result consistent with several studies in rats (Mohammed et al., 2010; Prem et al., 2012). Findings of this study clearly indicates that diabetic animals treated with *C. sinensis* and *C. paradisi* had a reduced blood glucose concentration when compared to the diabetic control animals, a result almost similar to reference drug glibenclamide (Table 3).

Present study showed significant blood glucose lowering effect by *C. sinensis* at all three doses, while there was also significant increase in plasma insulin levels. On the basis of these results, it could be postulated that *C. sinensis* induced hypoglycemic effect may be due to the presence of high contents of flavonoids and monoterpenes in *C. sinensis*. The hypo-glycemic effect of monoterpenes had been previously demonstrated by Tavafi et al. (2011). Monoterpenes may have insulin mimetic properties or may be able to induce insulin production from the surviving beta cells enough to facilitate glucose uptake from the blood.

C. paradisi showed highly significant increase in plasma insulin levels at 0.5 ml/kg. This effect may be due to the presence of vitamin C in *C. paradisi* which acts as strong antioxidant. This effect might be also due to the presence of naringin. Since naringin is reported to produce hypoglycemic effect due to its strong anti-oxidation property (Pari et al., 2010). Several flavonoids and terpenoids are present in *C. paradisi*. It is therefore, logical to conclude that the hypoglycemic effect of grapefruit juice (*C. paradisi*) may be due to flavonoids and/or terpenoids content of *C. paradisi* (Cerda et al., 1988). While there was highly significantly decrease in glucose level by CSP-2. This might be due to synergic effect of naringin in *C. sinensis* and polyphenols in *C. paradisi*. Moreover naringin and vitamin C had been reported to produce hypoglycemic and hypo-cholesterolemic effects due to their strong anti-oxidant properties (Pari and Suman, 2010). While polyphenols and vitamin C in *C. paradisi* may be responsible for these beneficial effects due to their strong antioxidant property (Violi et al., 2010).

All antioxidants may produce a synergistic effect which may provide strengthening to the B-cells of pancreas to release more insulin. Combined management with naringin and vitamin C had been demonstrated to ameliorate streptozotocin-induced diabetes in rats (Punithavathi et al., 2008). It has also been reported that hesperidin and naringin are useful for improving hyperlipidemia and hyperglycemia in type - II diabetic

animal models by partially regulating fatty acid and cholesterol metabolism and affecting gene expression of glucose-regulating enzymes (Jung et al., 2004; Jung et al., 2006). Though grapefruit juice has potent hypo-glycemic effects in experimental animals, however its use should be cautioned due to its enzyme inhibitory effects on the metabolism of many drugs (Bailey et al., 2000), which can lead to adverse effects from these medications (Gao et al., 2006).

Conclusion

From the results of the present study it may be concluded that combination of *C. sinensis* and *C. paradisi* is most effective in high doses, however further studies on more combination doses of *C. sinensis* and *C. paradisi* are required to reveal the role of these substances on reduction of blood glucose levels and combating diabetes mellitus. This study suggests that grapefruit juice and orange juice plays a good role in controlling the glucose level of experimental animals and can be applied clinically on patients with diabetes and lower cholesterol level.

Conflict of interest

There are no conflicts of interests

REFERENCES

American Diabetes Association. (2009). Standards of medical care in diabetes-2009. Diabetes care 32(1):S13-S61.

Armando C, Maythe S, Beatriz NP (1998). Antioxidant activity of grapefruit seed extract on vegetable oils. J Sci. Food Agric. 77:463-467.

Bailey DG, Dresser GK, Kreeft JH, Munoz C, Freeman DJ and Bend JR (2000). Grapefruit-felodipine interaction: effect of unprocessed fruit and probable active ingredients. Clin. Pharmacol. Ther. 68:468-477.

Behera B, Yadav D (2013). Current research on plants having antidiabetic potential: An overview. Research and Reviews. J. bot. Sci. 2(2):4-17.

Bernard T (2004). Guidelines on the care and use of animals for scientific purposes. National Advisory Committee for Laboratory Animal Research (NACLAR). Available at: www3.ntu.edu.sg/Research2/.../NACLAR-guide% Z0Lines.pdf.

Buscemi S, Rosafio G, Arcoleo G, Mattina A, Canino B, Montana M, Verga S, Rini G (2012). Effects of red orange juice intake on endothelial function and inflammatory markers in adult subjects with increased cardiovascular risk. Am. J. Clin. Nutr. 95(5):1089-1095.

Cerda JJ, Robbins FL, Burgin CW, Baumgatner TG, Rice RW (1988). The effects of grapefruit pectin on patients at risk for coronary heart disease without altering diet or lifestyle. Clin. Cardiol. 11:589-94.

Chiba H, Uehara M, Wu J, Wang X, Masuyama R, Suzuki K, Ishimi Y (2003). Hesperidin, a citrus flavonoid, inhibits bone loss and decreases serum and hepatic lipids in ovariectomized mice. J. Nutr. 133(6):1892-1897.

Free encyclopedia (2002). Grapefruit. Available at: http://en.wikipedia.org/wiki/Grapefruit.

Funaguchi N, Ohno Y, La BLB, Asai T, Yuhgetsu H, Sawada M, Takemura G, Minatoguchi S, Fujiwara T, Fujiwara H (2007). Narirutin inhibits airway inflammation in an allergic mouse model. Clin Exp.

Pharmacil. Physiol. 34(8):766-770.

Gao K, Henning SM, Niu Y, Youssefian AA, Seeram NP, Xu A, Heber D (2006). The citrus flavonoid naringenin stimulates DNA repair in prostate cancer cells. J. Nutr. Biochem. 17(2):89-95.

Ghanim H, Sia CL, Upadhyay M, Korzeniewski K, Viswanathan P, Abuaysheh S, Mohanty P, Dandona P (2010). Orange juice neutralizes the proinflammatory effect of a high-fat, high-carbohydrate meal and prevents endotoxin increase and Toll-like receptor expression. Am. J. Clin. Nutr. 91(4):940-949.

Giovannucci E, Rimm EB, Liu Y, Stampfer MJ, Willett WC (2002). A Prospective study of tomato products, Lycopene, and prostate cancer risk. J. Natl. Cancer Inst. 94:391-8.

Gorinstein S, Caspi A, Libman I, Lerner HT, Huang D, Leontowicz H, Leontowicz M, Tashma Z, Katrich E, Feng S, Trakhtenberg S (2006). Red Grapefruit Positively Influences Serum Triglyceride Level in Patients Suffering from Coronary Atherosclerosis: Studies *in Vitro* and in Humans. J. Agric. Food Chem. 54(5):1887-1892.

Graciela OR, Fredy Y, Betzabe SF, Lilibeth C (2003). Volatile fraction composition of Venezuelan sweet orange essential oil (*Citrus sinensis* (L.) Osbeck). Ciencia 11(1):55-60.

Ha SK, Park HY, Eom H, Kim Y, Choi I (2012). Narirutin fraction from citrus peels attenuates LPS-stimulated inflammatory response through inhibition of NF-κB and MAPKs activation. Food Chem. Toxicol. 50(10):3498-504.

Herbal Medicine (2000). Grapefruit. Available at: http://www.holistic-online.com/Herbal Med/_Herbs/h_grapefruit.htm.

Javascript AG (2002). Grapefruit, J. Altern Complement. Med. 8: 333-40.

Jung UJ, Lee MK, Jeong KS, Choi MS (2004). Hypoglycemic effects of hesperidin and naringin are partly mediated by hepatic glucose-regulating enzymes in C57BL/KsJ-db/db mice. J. Nutr. 134:2499–2503.

Jung UJ, Lee MK, Park YB, Kang MA, Choi MS (2006). Effect of citrus flavonoids on lipid metabolism and glucose-regulating enzyme mRNA levels in type-2 diabetic mice. Int. J. Biochem. Cell Biol. 38(7):1134–1145.

Kurowska EM, Spence JD, Jordan J, Wetmore S, Freeman DJ, Piché LA, Serratore P (2000). HDL-cholesterol-raising effect of orange juice in subjects with hypercholesterolemia. Am. J. Clin. Nutr. 72(5):1095-1100.

Liu Y, Heying E, Tanumihardjo SA (2012). History, global distribution, and nutritional importance of citrus fruits. Compr. Rev. Food Sci. Food Saf. 11(6):530-545.

Malviya N, Jain S, Malviya S (2010). Antidiabetic potential of medicinal plants. Acta. Pol. Pharm. 67(2):113-118.

Manach C, Morand C, Gil-Izquierdo A, Bouteloup-Demange C, Remesy C (2003). Bioavailability in humans of the flavanones hesperidin and narirutin after the ingestion of two doses of orange juice. Eur. J. Clin. Nutr. 57(2):235-242.

Mateljan G (2006). The world's healthiest foods-Grapefruit. Available at: http://www.whfoods.com/genpage.php?tname=foodspice&dbid25#healthbenefits.

Mohammed FA, Mohammed SK, Syed SG, Syeda S, Shaik RA, Shaik MA, Mohammed I (2010). Antidiabetic Activity of Vinca rosea Extracts in Alloxan-Induced Diabetic Rats. Int. J. Endocrinol. 841090:10-16.

Pari L, Suman S (2010). Antihyperglycemic and antilipidperoxidative effect of flavonoid naringin in streptozotocin-nictonamide induced diabetic rats. Int. J. Biol. Med. Res. 1(3):206-210.

Patel DK, Prasad SK, Kumar R, Hemalatha S (2012). An overview on antidiabetic medicinal plants having insulin mimetic property. Asian Pac. J. Trop. Biomed. 2(4):320-330.

Peluso MR (2006). Flavonoids attenuate cardiovascular disease, inhibit phosphodiesterase, and modulate lipid homeostasis in adipose tissue and liver. Exp. Biol. Med. 231(8):1287-1299.

Prem KS, Darshee B, Sudip B, Ramachandran AV (2012). Therapy with methanolic extract of *Pterocarpus marsupium* Roxb and *Ocimum sanctum* Linn reverses dyslipidemia and oxidative stress in alloxan induced type I diabetic rat model. Exp.Toxicol. Pathol. 64(5):441-448.

Punitha R, Vasudevan K, Monoharan S (2006). Effect of *Pongamia pinnata* flowers on blood glucose and oxidative stress in alloxan induced diabetic rats. Indian J. Pharmacol. 38:62-63.

Punithavathi VR, Anuthama R, Prince PS (2008). Combined treatment with naringin and vitamin C ameliorates streptozotocin-induced diabetes in male Wistar rats. J. Appl. Toxicol. 28(6):806-813.

Riaz A, Khan RA, Ahmed M (2013). Glycemic response of Citrus Limon, Pomegranate and their combinations in Alloxan induced diabetic rats. Aust. J. Basic Appl. Sci. 7(10):215-219.

Rogerio AP, Sá-Nunes A, Faccioli LH (2010). The activity of medicinal plants and secondary metabolites on eosinophilic inflammation. Pharmacol. Res. 62(4):298-307.

Rohilla A, Ali S (2012). Alloxan induced diabetes: Mechanism and effects. Int. J. Res. Pharmaceut. Biomed. Sci. 3(2):819-823.

Roza JM, Xian-Liu Z, Guthrie N (2007). Effect of citrus flavonoids and tocotrienols on serum cholesterol levels in hypercholesterolemic subjects. Altern. Ther. Health Med. 13(6):44.

Samreen R (2009). Diabetes mellitus. Sci. Res. Essay 4(5):367-373.

Sawalha S, Arráez-Román D, Segura-Carretero A,Fernández-Gutiérrez A (2009). Quantification of main phenolic compounds in sweet and bitter orange peel using CE–MS/MS. Food Chem. 116(2):567-574.

Tahrani AA, Piya MK, Kennedy A, Barnett AH (2010). Review Glycemic control in type 2 diabetes: targets and new therapies. Pharmacol. Ther. 125(2):328-61.

Tavafi M, Ahmadvand H, Tamjidipoor A, Delfanc B. Khalatbarid AR Satureja khozestanica (2011). Essential oil ameliorates progression of diabetic nephropathy in uninephrectomized diabetic rats. Tissue Cell J. 43:45-51.

Violi F, Pignatelli P, Basili S (2010). Nutrition, Supplements and Vitamins in Platelet Function and Bleeding. Circulation 121:1033-1044.

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- *Journal of Parasitology and Vector Biology*
- *Journal of Pharmacognosy and Phytotherapy*
- *Journal of Toxicology and Environmental Health Sciences*