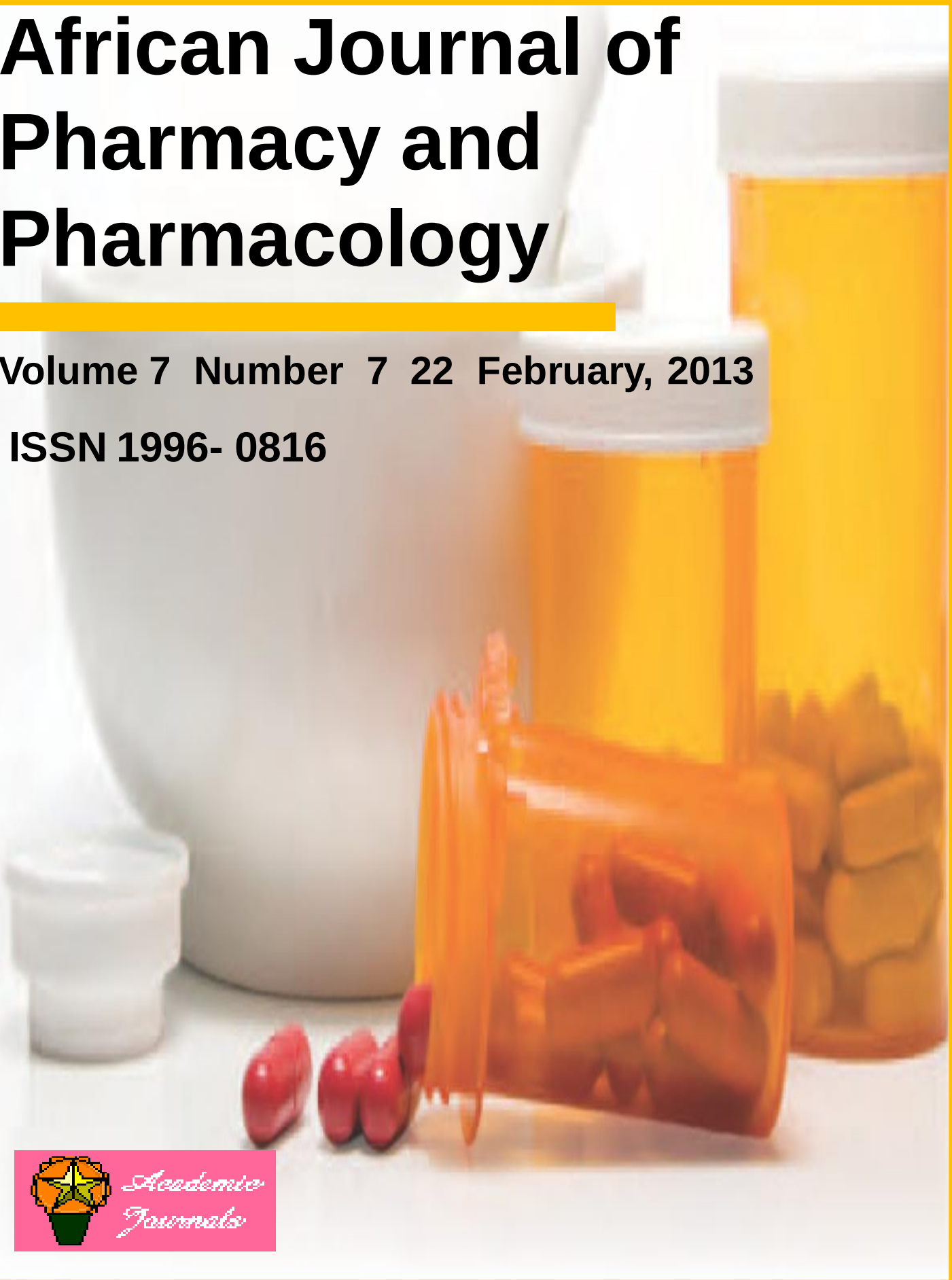
African Journal of Pharmacy and Pharmacology

Volume 7 Number 7 22 February, 2013

ISSN 1996- 0816



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Charnley AK (1992). Mechanisms of fungal pathogenesis in insects with particular reference to locusts. In: Lomer CJ, Prior C (eds) Pharmaceutical Controls of Locusts and Grasshoppers: Proceedings of an international workshop held at Cotonou, Benin. Oxford: CAB International, pp 181-190.

Jake OO (2002).Pharmaceutical Interactions between Striga hermonthica (Del.) Benth. and fluorescent rhizosphere bacteria Of Zea mays, L. and Sorghum bicolor L. Moench for Striga suicidal germination In Vigna unguiculata . PhD dissertation, Tehran University, Iran.

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Research Articles

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Available online at http://www.academicjournals.org/AJPP
DOI: 10.5897/AJPPX12.005
ISSN 1996-0816 ©2013 Academic Journals

Review

Toxicity of exhaust nanoparticles

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Accepted 14 January, 2013

The environmental problems in India are growing rapidly. The increasing economic development and a rapidly growing population that has taken the country from 300 million people in 1947 to more than one billion people today, is putting a strain on the environment, infrastructure, and the country's natural resources. Industrial pollution, soil erosion, deforestation, rapid industrialization, urbanization, and land degradation are all worsening problems. Over-exploitation of the country's resources, be it land or water, and the industrialization process, has resulted in environmental degradation of resources. Environmental pollution is one of the most serious problems facing humanity and other life forms on our planet today. The rapid growth in motor vehicle activity and rapid industrialization is contributing to high levels of urban air pollution. The population is mainly exposed to high air pollution concentrations; where motor vehicle emissions constitute the main source of fine and ultrafine particles, having a serious impact on our urban air quality and public health. Studies showed that the composition of atmospheric particulate matter has a great impact on human health. In this study, we have reviewed the toxicity of vehicle exhaust, especially diesel exhaust nanoparticles and the associated health problems.

Key words: Pollution, diesel exhaust, toxicity, air pollutants, nanoparticles.

INTRODUCTION

The World Health Organization (WHO) estimates that about two million people die prematurely every year as a result of air pollution (Table 1), while many more suffer from breathing ailments, heart disease, lung infections and even cancer. Fine particles or microscopic dust from coal or wood fires and unfiltered diesel engines are rated as one of the most lethal forms or air pollution caused by industry, transport, household heating and cooking. The main four sources of air pollution are emissions from vehicles, thermal power plants, industries and refineries. The source of indoor air pollution is mainly due to kitchen stoves in rural areas. In the 2007, the Blacksmith Institute listed the top ten polluted areas in the world as Azerbaijan, China, India, Peru, Russia, Ukraine, and Zambia (Blacksmith Institute). Vehicle emissions are responsible for 70% of the country's air pollution. Air pollution from vehicle exhaust and industry is a worsening problem in India. Exhaust from vehicles has increased eight-folds over a period of twenty years; industrial pollution has risen four times over the same period.

The economy has grown two and a half times over the past two decades but pollution control and civic services have not kept pace. Air quality is worst in big cities like Kolkata, Delhi, Mumbai, Chennai, etc. Bangalore holds the title of being the asthma capital of the country. Studies estimate that 10% of Bangalore's 60 lakh population and over 50% of its children below 18 years suffer from air pollution- related ailments. In Chennai and Mumbai, exhaust from vehicles, dust from construction debris, industrial waste, and burning of municipal and garden waste are all on the rise; so are respiratory diseases, including asthma. At least six of the 10 top causes of death are related to respiratory diseases (The Times of India).Particulate matter (soot, PM) is the major air pollutant in the atmosphere. It is a complex mixture of organic and inorganic substances present in the atmosphere as both liquids and solids. Coarse particulates can be regarded as those with a diameter greater than 2.5 µm

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Table 1. Common air pollutants and their effects.

Pollutant source	Pollutant	Adverse effect	Reference
Smoke, dust	Particulate matter (PM)	Pulmonary problems, effect on immune system	Batsura et al. (1981), Mott et al. (2002) and Taylor (2002)
Chemical industries	Pesticides	Cancer, neurotoxicity	Antonini et al. (2006) and Gold et al. (2001)
Volcanos, rocks, water, soil	Radon, nano-sized particles	Pulmonary toxicity Podoconiosis, Kaposi's Sarcoma	Fuller (2005), Montella et al. (1997) and Mott et al. (2002)
Air conditioner, refrigerators, fire extinguishers	Chlorofluorocarbons, nanoparticles, particulate matter	Ozone effects	Buzea et al. (2007), Mott et al. (2002) and Sapkota et al. (2005)
Vehicle Exhaust	Sulphur dioxide (SO_2)	Pulmonary toxicity, affects vision	Donaldson (2004), Knox, (2005), Peters (2005), Risom et al. (2005) and Vermylen et al. (2005)
Motor emissions	Carbon dioxide (CO_2), particulate matter	Pulmonary toxicity, climatic changes, myocardial damage	Buseck et al. (1999), Peters et al. (2001), Pope et al. (2002)
Incomplete combustion from automobiles	Carbon monoxide (CO)	Neurotoxicity, affects visibility	Donaldson et al. (2005)
Burning Fuels	Nitrogen oxides (NOx)	Respiratory health	Hogan et al. (2004)
Coal mining, natural gas systems	Methane	Pulmonary toxicity, climatic changes	Antonini et al. (2006), Borm et al. (2002), Shah, (1998) and Weiss (2005)
Dust, soil, mining units	Lead, manganese	Organ toxicity, neurotoxicity	Borm et al. (2002) and Gustavsson et al. (1988)
Hazardous waste	Mercury	Neurotoxicity, immunotoxicity	Shah, (1998) and Gatti et al. (2004)
Paint, cosmetics	Propellants	Effects on ozone layer	Gatti et al. (2004), Lademann et al. (1999), Oberdorster et al. (2005), Takenaka et al. (2001) and Rehn et al. (2003)
Vehicle and industrial emissions	Ozone (O_3), lead, cadmium	Pulmonary toxicity	Kocbach et al. (2006) and Waalkes, (2003)
Old cosmetics, vehicle emissions	Aerosols	Organ toxicity	Donaldson et al. (2004), Kocbach et al. (2006), Peters et al. (2001) and Takenaka et al. (2001)
Building materials, building demolition	Asbestos fibres, lead, glass, wood, paper	Respiratory health	Fireman et al. (2004) and Stefani et al. (2005)
Agriculture	Ammonia	Cytotoxicity	Buzea et al. (2007)
Hydrocarbons	Diesel exhaust emissions	Pulmonary and organ toxicity	Donaldson et al. (2005), Hoet et al. (2004), Risom et al. (2004) and Vermylen et al. (2005)

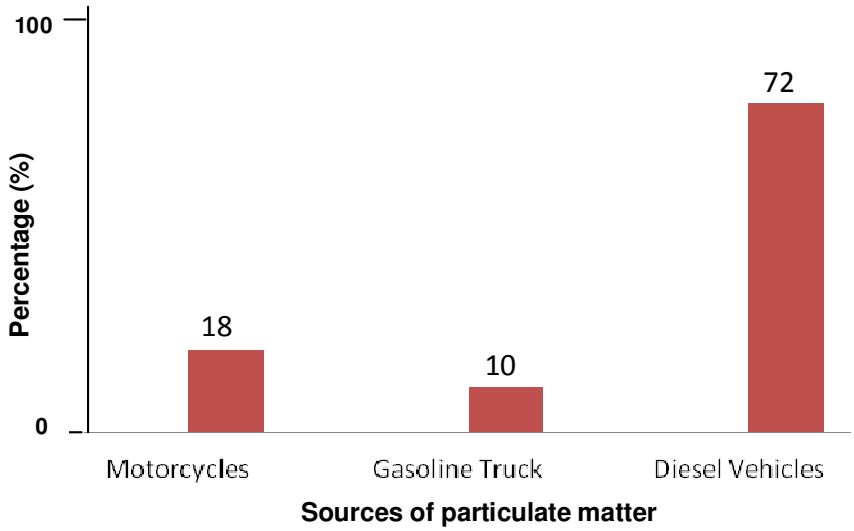

Figure 1. Different sources of particulate matter 2.5 µm (PM 2.5).

and fine particles less than 2.5 µm (Figure 1). The most significant primary sources of particulate matter are motor exhaust (25%), non-combustion processes (24%), industrial combustion plants and processes (17%), commercial and residential combustion (16%) and public power generation (15%) processes (Peters et al., 2006). Inhalable particles, particularly fine particles, have the greatest demonstrated impact on human health. Their small size allows them to get deep into the lungs and from there they can reach or trigger inflammation in the lung, blood vessels or the heart, and perhaps other organs. Studies have linked PM exposure to health problems and environmental issues. Studies showed that the composition of atmospheric particulate matter has a great impact on human health (Chan and Lippmann, 1980; Braunfahrlander et al., 1992; Dockery and Pope, 1994; Pope et al., 1995; Berico et al., 1997).

Motor exhaust emissions is a complex mixture of gases and particulate matter. The particles consist of a core of elemental carbon (EC) to which organic compounds formed during the combustion is adsorbed. Traces of metal compounds and sulphates are also present in the particulate fraction. At formation, the particles are very small, with an aerodynamic diameter of less than 0.1 µm, later they aggregate and form larger particles. Most of these are still smaller than 1 µm (IARC, 1989). These particles can penetrate deep into the respiratory system, and studies indicate that the smaller the particle, the larger the health impacts.

In Chennai (India), vehicles are the major source of air pollution. It is estimated that vehicular emissions contribute more than 300 tons/day of pollution load into the city atmosphere. As at 1st January, 2008, the vehicle population in the city was estimated as Car/jeep (3.0 lakhs); two wheeler (2.0 lakhs). According to the Comprehensive Transportation Study of the Chennai Metropolitan Development Authority (CMDA), levels of suspended particulate matter (SPM) in the city ranged from 274 to 1,470 mg/m^3, which is much higher than the World Health Organization's (WHO) prescribed limit of 200 mg/m^3 (Durga et al.,2012). It goes on to say that about 70% of the pollution load is vehicular. Studies by Bathmanabhan et al. (2010) measured average particulate matter PM10, PM2.5 and PM1 concentrations near an urban roadway in Chennai city, India. Results indicated that highest PM concentrations were observed during weekday's peak hour traffic and lowest PM concentrations were found during trickle traffic (afternoon and night-time).

Combustion Derived Nanoparticles (CDNPs) are defined as primary particles with at least one dimension < 100 nm, while ultrafine particles are defined as particles < 100 nm in all dimensions and are commonly produced by combustion processes (Avakian et al., 2002; Lighty et al., 2002). Nanoparticles have the ability to escape from the site of deposition in lungs and reach other organs through circulation, and are capable of causing inflammation (Donaldson et al., 2004). Diesel soot, welding fume, carbon black and coal fly ash are the major sources of CDNPs.

Diesel-powered vehicles contribute 72% and gasoline/petrol vehicles contribute 10% to particulate matter on roads. Diesel vehicles produce ~2 to 40 times more particles than petrol-powered vehicles, depending on the type of diesel fuel and the detailed construction of the engine (IARC, 1989). Combustion of petrol in modern vehicles produces less nitrogen oxides (NOx) and particulates than diesel but more than Liquid Petroleum Gas (LPG). LPG vehicles tend to produce lower levels of emissions across a range of air pollutants compared to

petrol, biodiesel and diesel fuelled vehicles. Toxicologists can more readily study the components of PM and there has been considerable amount of research demonstrating the toxicity of combustion-derived particles such as diesel soot (Dybdahl et al., 2004; Hirano et al., 2003), welding fume (McNeilly et al., 2004), carbon (Renwick et al., 2004). They mediate a range of adverse effects in the lungs and other organs, and warrant further research.

NANOTOXICOLOGY

Nanotoxicology is a branch of bio-nanoscience which deals with the study and application of toxicity of nanomaterials. Some nano-particles, depending on their composition and size, can produce irreversible damage to cells by oxidative stress and/or organelle injury. The toxicity of nanoparticles depends on various factors, including size, aggregation, composition, crystallinity, surface functionalization etc. In addition, the toxicity of any nanoparticle to an organism is determined by the individual's genetic complement (Buzea et al., 2007).

Nanotoxicology publication statistics

The total number of papers on toxicity is seen to increase in the graph (Figure 2) that has been published in the ISI web of knowledge database, but till the year 2005, only around 500 toxicological articles has been published. This clearly indicates that more studies are required in the area of nanotoxicology; especially, toxicology of environmental nanoparticles need more concern.

About 60% of nanoparticles in the environment are due to road transport, and a further 27% come from other combustion processes such as power stations. It is the air-borne nanoparticles that are of most concern to human health as it has been shown that increase in the levels of ultra-fine particulates in the air which are less than 10 micrometre in diameter can be considered as being responsible for the increased respiratory and cardiac diseases, and there is increasing evidence that nanoparticles within this fraction can penetrate the lung, causing inflammation and can spread to other organs within the body (http://www.nanoforum.org).

DIESEL AND PETROL ENGINES

Diesel fuel used in diesel engines such as compression-ignition diesel engine was invented by Rudolph Diesel in 1892 as an alternative to the spark-ignition gasoline engine (Gilman, 2002). Petrol engine also known as gasoline engine is a type of internal combustion engine designed to run on petrol (gasoline) and similar volatile fuels. It differs from a diesel engine in the method of mixing the fuel and air, and in the fact that it uses spark plugs to initiate the combustion process. In a diesel engine, only air is compressed (and therefore heated), and the fuel is injected into the now very hot air at the end of the compression stroke, and self-ignites. In a petrol engine, the fuel and air are usually pre-mixed before compression. Diesel fuel is a middle distillate of petroleum which contains paraffin's, alkenes and aromatics.

The engine's popularity expanded because it had excellent fuel economy and durability and it required less maintenance, and the fuel was used in mass transportation vehicles such as trucks, buses, and trains. Diesel fuel and the products of its combustion represent one of the most common toxins to which people living in both urban and rural areas of the world are exposed. Diesel engines are typically separated according to their service requirements, light-duty or heavy-duty. The total particulate emission concentration from light-duty diesel engines is much smaller than from heavy-duty diesel engines. However, the total particulate matter emitted from diesel engines is much higher than from petrol engines and LPG engines (IPCS, 1996).

Composition of diesel exhaust and petroleum exhaust

Our previous study demonstrated that the collected vehicle exhaust samples contained carbon aggregates consisting of tens to thousands of primary carbon particles and mineral particles. The Petrol Exhaust Particles (PEPs) contained slightly larger size particles (Figure 3) compared to the Diesel Exhaust Particles (DEPs) (Figure 4). Both samples contained particles of nano-size. The elemental analysis for the two samples, PEPs and DEPs, was performed using the Scanning electron microscopy with energy dispersive X-ray spectroscopy (SEM-EDX) technique. Both the samples contained carbon as the major element. The percentage of carbon in PEPs was comparatively more than in DEPs. Besides carbon, the PEPs contained elements like aluminium, silica, lead, sulphur, calcium and iron in trace amounts compared to the DEPs, which contained only carbon, oxygen and sulphur (Durga et al., 2012).The inorganic fraction of the particulate phase of diesel fuel combustion emissions primarily consists of small elemental carbon particles. The organic and elemental carbon accounts for approximately 80% of the total particulate matter mass. The remaining 20% is composed of sulfate (mainly sulfuric acid) (Pierson et al., 1983) and some inorganic additives and components of fuel and motor oil. The organic compounds identified in diesel exhaust emissions contain hydrocarbons and hydrocarbon derivatives. Diesel exhaust particulates are capable of adsorbing relatively large amounts of organic material because of their high surface area.

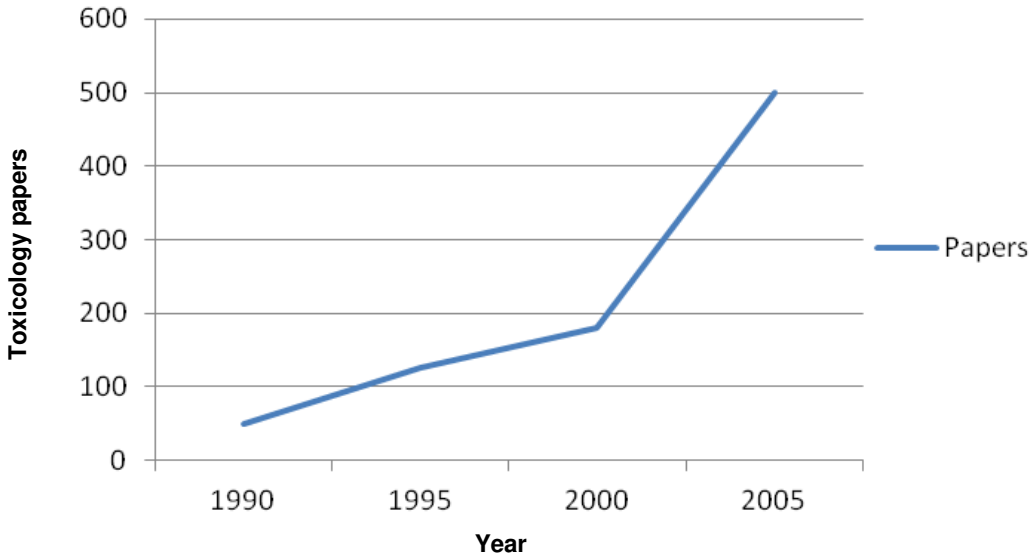

Figure 2. Publication statistics of toxicology papers.

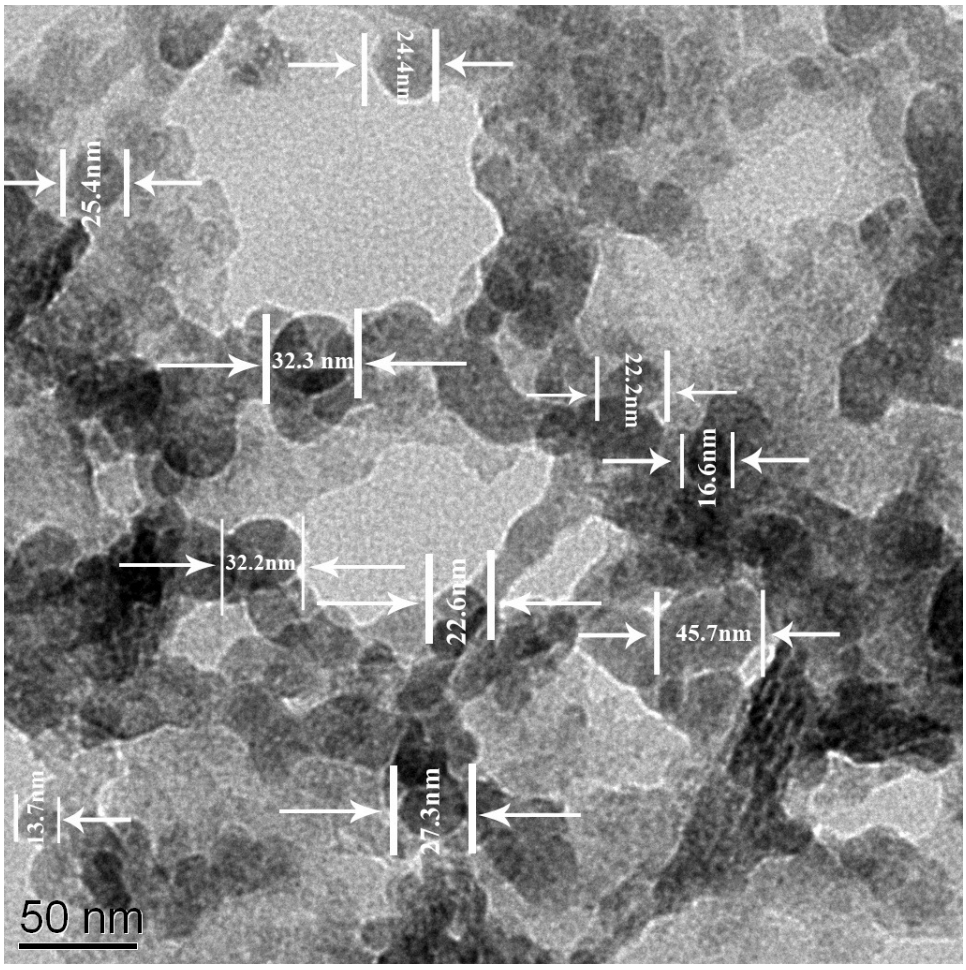

Figure 3. TEM micrograph of petrol sample showing carbon aggregates of various sizes.

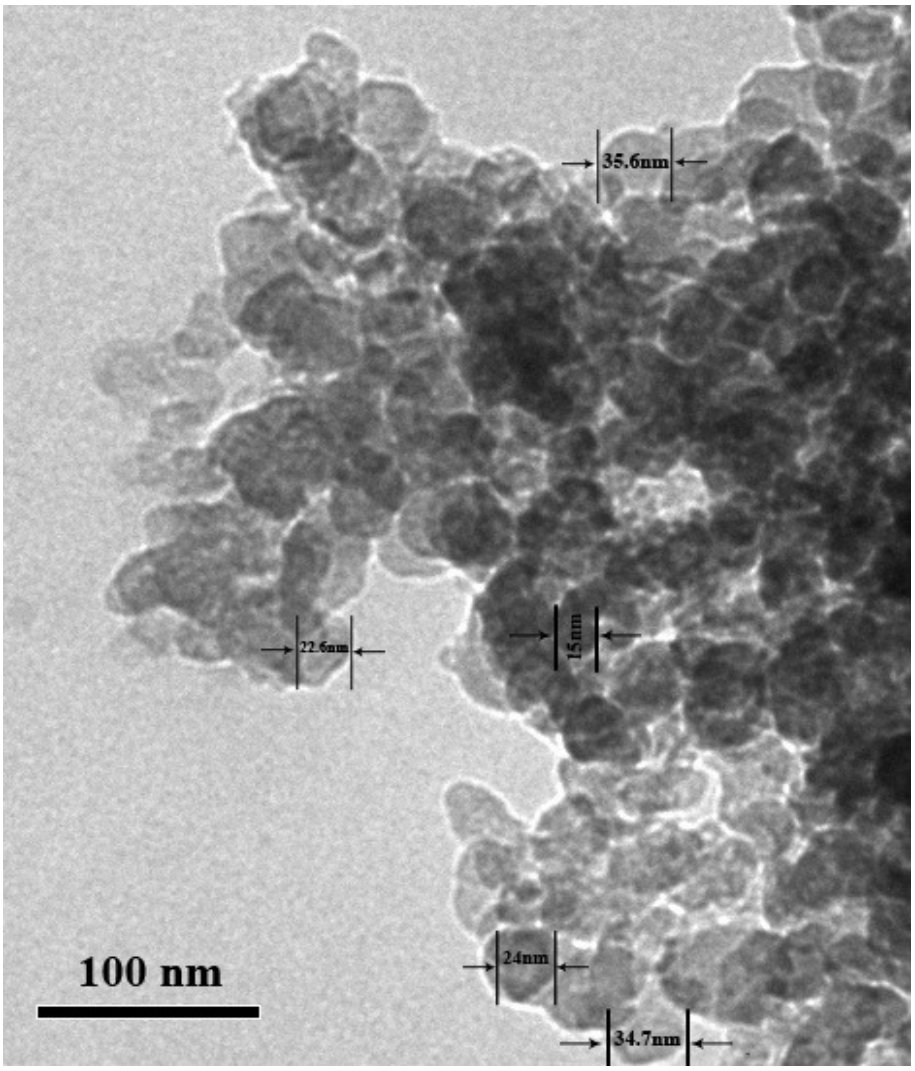

Figure 4. TEM micrograph of diesel sample showing carbon aggregates of various sizes.

Vehicle exhaust of clinical significance

The significant sources of vehicle exhaust include roads and highways, airports, railway stations, truck stops or distribution points, construction sites, tunnels, gas stations, vehicle repair and maintenance shops, bridges, parking lots, bus depots, fire stations, boat harbors and mines (Irina et al., 2008).

IN VITRO TOXICITY STUDIES OF DIESEL EXHAUST PARTICLES (DEPS)

DEP caused oxidative stress in a number of models *in vitro* such as oxidation of low density lipoprotein (LDL) (Ikeda et al., 1995) and in exposed epithelial cells (Li et al., 2000; Hirano et al., 2003). The component responsible for the oxidative stress and subsequent pro-inflammatory signaling is principally the organic fraction (Bonvallet et al., 2001), although transition metals may also be involved (Ball et al., 2000). Studies on broncho-epithelial cell lines have shown increase in levels of cytokines such as IL-8 and granulocyte macrophage stimulating factors (Doornaert et al., 2003).

Le Prieur et al. (2000) studied the toxicity of diesel engine exhausts in an *in vitro* model of lung slices in biphasic organotypic culture, and the results showed induction of a proinflammatory and apoptotic responses. Studies on toxicity of diesel exhaust particles were done in Human Eosinophilic cells; results indicated that DEPs induce monocyte chemoattractant protein-1 (MCP-1) and interleukin-8 (IL-8) production by up-regulating nuclear factor -kappa B (NF-κB) activity and also promotes eotaxin-induced chemotaxis. A possible association between chronic DEP exposure and Parkinson's disease has been explored because DEPs have been shown to

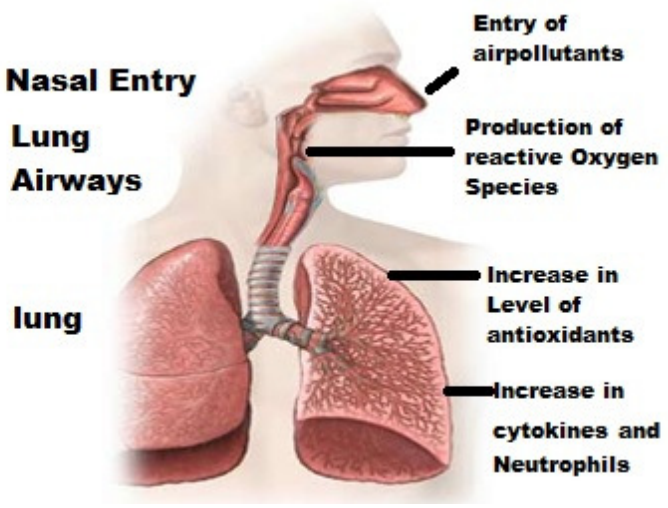

Figure 5. Effect of air pollutants on the lungs.

decrease the number of dopaminergic neurons in the brain tissue of mice (Block et al., 2004). Hartz et al. (2008) studied the *in vitro* effect of blood-brain barrier proteins on exposure to diesel exhaust particles in rat brain capillaries and as a result observed up-regulation in P-glycoprotein, Multi-drug resistance associated protein 1,2 and 4, presence of cytokines and reactive oxygen species in brain capillaries. In our previous study, the cytotoxicity of vehicle exhaust nanoparticles were investigated (Durga et al., 2012). The Petrol Exhaust Nanoparticles (PENPs) and the Diesel Exhaust Nanoparticles (DENPs) were screened for their cytotoxicity on HT29, A549, MDA MB 231, HEP 2 and VERO cell lines at various concentrations of up to 1,000 µg. The IC_{50} (Concentration at which growth of 50% of the cell lines is inhibited) values for DENPs and PENPs in monkey kidney cell lines (VERO) and colon cancer cell lines (HT29) were found to be 250 and 125 µg/ml, respectively. Both the nanoparticles exhibited the same level of effect on the above two cell lines. Whereas in contrast to this, in lung cancer cell lines (A549) and larynx cancer cell lines (HEP2), the PENPs were found to be toxic at low concentrations of 62.5 µg in comparison to the DENPs which were toxic to A549 only at 250 µg and HEP2 at 125 µg.

Studies on breast cancer cell lines (MDA MB231) indicated that DENPs were more toxic at low concentrations of 62.5 µg than PENPs, which were found to be toxic only at 125 µg. Hence, the studies indicate different levels of cytotoxicity of these two particles on five different cell lines. It was observed that the cell viability was significantly reduced in a dose-dependent manner after the cell lines were treated with the vehicle exhaust nanoparticles using the Tetrazolium dye (MTT) assay.

Accurately assessing the toxicity and safety of these vehicle exhaust nanoparticles to human health is of utmost importance. Toxicity data generated in this study will be potentially useful to assess human risk exposure to these nanoparticles. Future studies should be focused on investigating the potential risk of these nanoparticles to human health at the microscopic cellular level by implementing appropriate *in vivo* toxicity method to reveal the general mechanisms of organ toxicity. More studies will be carried out in detail for organs like the brain and kidneys. The results of the present study indicate that these nanoparticles can be toxic to normal cell lines and to the cancerous cell lines at varying levels. Thus, the *in vivo* studies should be carried out to study in detail the vehicle exhaust particle-mediated toxicity.

IN VIVO TOXICITY STUDIES OF DIESEL EXHAUST PARTICLES

The human skin, intestinal tract and lungs are constantly in contact with the environment. The lungs and the intestinal tract allow the transport of water, oxygen or nutrients by the method of active or passive diffusion, whereas the skin acts as a strong barrier. The lungs and the intestinal tract are more prone to the entry of nanoparticles inside the human body (Hoet et al., 2004). Once these particles enter the circulatory system, they can cross various organ barriers (Table 2) and affect other vital organs (Figure 5). Inflammation, oxidative stress and carcinogenicity are the major effects of nanoparticles.

Lung toxicity

The lungs being the main site of gas exchange have two important parts; airways for the transportation of air and the alveoli for gas exchange. Human lungs contain about 2,300 km of airways and 300 million alveoli. In human adults, the internal surface area of the lungs is 140 m^2. The large surface area of the alveoli makes the alveoli less protected against environmental damage when compared to the airways, hence the lung is the primary site of entry for the inhaled nanoparticles (Hoet et al., 2004; Buzea et al., 2007). The main mode of deposition of these nanoparticles is diffusion, due to displacement when they collide with air molecules. The lungs mainly receives nanoparticles of size between 10 to 20 µm, while the naso-pharyngeal region captures particles of size less than 10 µm as shown in the figure (Oberdorster et al., 2001). DEP causes inflammation in rat lungs (Miyabara et al., 1998; Tsurudome et al., 1999) and in human lungs (Nordenhall et al., 2000) following short-term, high level exposure.

Evidence of the oxidative properties of DEP *in vivo* is shown by increased level of 8 OH dG (8 hydroxy deoxyguanine), the oxidative adduct of hydroxyl radical in the lungs of rats following exposure, and in cells in culture treated with DEP (Ichinose et al., 1997; Arimoto et al., 1999). Studies have shown that DEP exposure may be

Table 2. Nanoparticles and related toxicity.

S/No.	Mode of entry of nanoparticle	Toxicity involved	Related diseases	References
1	Lung through inhalation	Pulmonary toxicity	Asthma, bronchitis, emphysema, lung cancer, and neurodegenerative diseases, such as Parkinson's and Alzheimer's diseases	Borm et al. (2004, 2006), Oberdorster et al. (2005) and Peters et al. (2006)
2	In circulation	Organ toxicity	Arteriosclerosis, blood clots, arrhythmia, heart diseases, and ultimately cardiac death	Brown et al. (2002), Chen et al. (2006), Geiser et al. (2005), Liu et al. (2006) and Vermylen et al. (2005)
3	In the gastrointestinal tract	Intestinal toxicity	Crohn's disease and colon cancer	Buzea et al. (2007), Gatti et al. (2004), Oberdorster (2004) and Takenaka et al. (2001)
4	Immune system	Immunotoxicity	Autoimmune diseases, such as systemic lupus erythematosus, scleroderma, and rheumatoid arthritis	Ng et al. (2004), Peters et al. (2006), Noonan et al. (2006) and Pfau et al. (2005)
5	Through skin	Dermal toxicity	Dermatitis	Borm et al. (2006), Oberdorster et al. (2005), Tinkle et al. (2003) and Toll et al. (2004)

be the cause of diseases such as asthma in patients (Proietti et al., 2003; Takizawa, 2004). The study subjects were exposed to DEP of concentration 300 pg/m^3, results indicated marked neutrophilic inflammatory response in the airways followed by increases in blood neutrophil and platelet counts (Holgate et al., 2003). Visits to the emergency department for pulmonary complaints have been shown to increase during periods of severe air pollution (Wilson et al., 2005).

Studies by Diaz-Sanchez et al. (2000) showed that diesel exhaust particles can cause degranulation of mast cells and release histamine which further can cause chronic cough, sinusitis, pharyngitis and laryngitis (Groneberg-Kloft et al., 2006). Many of the hydrocarbon molecules emitted by diesel engines are quite toxic to the lungs. Studies in Mexico City on humans by Churg et al. (2003), showed that ultrafine particles were embedded in the airway mucosa. Workers in enclosed spaces such as mines and ships are especially at risk from DEP-induced pulmonary disease (Jorgensen and Svensson, 1970).

TOXICITY OF THE HEART

Diesel exhaust particles also induce cardiac effects such as heart rate variability and ventricular arrhythmia. A significant decrease in left-ventricular systolic pressure and an increase in left-ventricular end-diastolic pressure were observed in animal models (Wold et al., 2006; Anselme et al., 2007). Myocardial damage due to superoxide radical induced by DEP was studied (Okayama et al., 2006). Cardiac effects due to diesel exhaust particles results in coronary vasoconstriction, transient thrombus formation, carbon monoxide exposure, and altered myocardial energetic (Mittleman, 2007).

A recent study of postmenopausal women concluded that there was an increased risk of cardiovascular disease with long-term exposure to air pollution containing diesel (Miller et al., 2007). Onset of myocardial infarction was seen associated with one hour exposure to vehicle traffic containing diesel exhaust particles (Peters et al., 2004). DEP exposure studies in guinea pig models proved them to be cardiotoxic (Minami et al., 1999). Effect of DEP on the synthesis of Immunoglobin E and on the release of histamine was studied (Mamessier et al., 2006). The relationship between atherosclerosis and air pollution has also been investigated. The study rabbits were exposed to particulate matter of size 10 microns for 4 days, followed by histological examination of bone marrow lesions (Suwa et al., 2002).

DEP AND HYPERTENSION

Several studies demonstrated the relationship between DEP exposure and hypertension. Short-term inhalation of fine particulate air pollution and ozone, at concentrations that occur in the urban environment, causes acute conduit artery vasoconstriction. 25 healthy adults underwent exposure to 2 h inhalation of ≈150 µg/m^3 of concentrated ambient fine particles along with ozone (Brook et al., 2002). At levels encountered in an urban environment, inhalation of dilute diesel exhaust impairs

two important and complementary aspects of vascular function in humans: the regulation of vascular tone and endogenous fibrinolysis. These important findings provide a potential mechanism that links air pollution to the pathogenesis of atherothrombosis and acute myocardial infarction (Mills et al., 2005).

RENAL TOXICITY

Acute renal failure (ARF) is increasingly becoming more frequent and is associated with high costs and adverse clinical outcomes, including excess mortality, increased length of hospital stay, and the requirement for chronic dialysis in survivors.

Studies showed the potentiating effect of diesel exhaust particles (DEP) in an animal model of ARF induced by a single ip injection of cisplatin (CP) in rats, followed by the intra-tracheal administration of DEP of concentration 0.5 or 1 mg/kg. The results indicated increased serum concentrations of urea and creatinine and the reduced glutathione (GSH) concentration and superoxide dismutase activity in renal cortex. Also, renal tubular necrosis; increased urine volume, protein concentrations, and N-acetyl-*b*-D-glucosaminidase (NAG) activity, followed by decreased urine osmolality was seen (Nemmar et al., 2010). The changes in the above parameters can be considered as evidences for renal toxicity.

BRAIN TOXICITY

Association between exposure to diesel exhaust and neuro-inflammation was investigated in study rats. Rats were exposed to DEP through a nose only exposure chamber for 6 h a day, 5 days a week, for 4 weeks. Pro-inflammatory markers such as cytokines were studied in different brain sections and compared.

Tumour necrosis factor alpha and Interleukin 1 were found to increase in the striatum region of the brain (Miriam et al., 2010).

Another study group demonstrated that brain inflamemation was induced by air pollution from different sources and as a result, histopathology changes similar to those seen in patients with Alzheimer's disease were observed (Garciduenas et al., 2004). Learning ability, coordination, memory, and judgment in both children and adults were affected due to chronic exposure to diesel exhaust particles (Margai and Henry, 2003). Studies demonstrated slowness of response, memory loss and disordered sleep which is suggestive of neurobehavioral impairment in workers whose occupations involved significant indoor diesel exhaust exposure (Kilburn et al., 2000).

DEP AND INFERTILITY

Many studies have shown a correlation between diesel exhaust exposure and premature births, low birth weight in infants and elevated infant mortality rates (Kim et al., 2004; Parker et al., 2005; Dolk at al., 2003). Another study group demonstrated decrease in adult sperm production and sperm motility in animal models (Watanabe, 2005) and aberration of sex hormone production in chronically exposed female rats. Exposure of DEP to pregnant rats demonstrated negative effects (Fredricsson et al., 1993). Isolation of 4-nitrophenaol (PNP) from DEP demonstrated that DEP has estrogenic and antiandrogenic activities *in vivo*, leading to sterility (Li et al., 2006). Investigations have also reported that the presence of PNP in the environment may be one of the factors responsible for the increasing incidence of sterility in humans and animals.

DEP AND CARCINOGENICITY

In 1989, the International Agency for Research on Cancer concluded that there is sufficient evidence for the carcinogenicity of diesel exhaust in experimental animals but limited evidence for carcinogenicity in humans. Animal studies showed direct DNA damage and carcinogenesis due to DEP exposure (Dybdahl et al., 2004) as a result of generation of reactive oxygen species. Mutation and DNA strand breakage were also observed due to formation of Poly Aromatic Hydrocarbon-DEP adducts (Li et al., 2006). Lung cancer has been reported by studies on railway workers, smokers as well as non-smokers (Parent et al., 2007). Gustavsson et al. (1993) reported that workers exposed to combustion products had a higher incidence of esophageal cancer. The possible relationship between exposure to DEP and multiple myeloma was investigated (Lee et al., 2003). In a study by Guo et al. (2002) human exposure to DEPs was associated with a higher risk of ovarian cancer but not with esophageal, testicular, or urinary tract cancers or leukemia.

DEP AND HEPATOTOXICITY

Diesel exhaust contain dozens of liver damaging poisons such as lead, sulfur and nitrogen oxides, acetaldehyde, cadmium, and peroxyacetylnitrile. The ability of liver microsomes to oxidize benzo alpha pyrene on chronic exposure to diesel exhaust particles was studied. Results showed that the microsomes were unable to generate polar metabolites from benzo alpha pyrene on exposure to highest concentration of DEP. Further studies with liver microsomes showed that after several months of exposure, there was no evidence for the induction of either cytochrome P-450 or cytochrome P-448 (Navarro et al., 1981). Studies indicated that DEP exposure and mortality due to atherosclerosis and cirrhosis of the liver are directly related. The main factor involved in atherosclerosis is Peroxisome Proliferator Activated Receptor (PPAR α). Studies were investigated whether nanoparticle-

rich diesel exhaust (NR-DE) affects the liver and how PPARα is involved in the NR-DE induced effects. The results indicated that NR-DE induced hepatic inflammation and dyslipidemia (Ito et al., 2011).

DEP AND PLACENTAL TOXICITY

Studies investigated the effects of DEP exposure on DNA adduct formation, and DNA deletions and levels of oxidative DNA damage during the embryonic development in mice. Oral exposure to various concentrations of DEP resulted in black pigmented spots in the retinal pigment in the epithelium of offspring mice. Results also revealed that transplacental exposure to DEP showed increase in frequency of DNA deletions and other genetic alterations in the mice offsprings (Reliene et al., 2005). Recent studies suggest that diesel exhaust possesses endocrine activity and therefore may affect reproductive outcome (Hougaard et al., 2008). Studies involving *In utero* exposure to DEP revealed decrease in weight gain of DEP exposed offspring compared to the control groups. This difference increased significantly during lactation. The other biomarkers of placental toxicity were found to be similar. mRNA levels of inflammatory cytokines IL-2, IL-5, IL-12 alpha, IL-12 beta increased in placentas exposed to DEP. IL-5 mRNA was markedly increased in DEP-exposed placentas, although levels were barely detectable in control placentas. IL-6 mRNA expression was increased approximately 10-fold in placentas exposed to DEP. It has been studied that expression of mRNA encoding proteins involved in immune function in the placenta is increased during fetal absorption in mice (Ayaha et al., 2005).

POTENTIAL TREATMENT FOR TOXICITY

The particle component of air pollutant called the particulate matter is responsible for deaths of more than 5 lakh people every year (US Environmental Protection Agency); the major composition being vehicle exhaust particles which mainly include DEP. The DEP contain dozens of organ damaging poisons. It consists of a carbon core onto which different compounds of organic nature are attached. The main mechanism involving the toxicity of diesel exhaust particle include the depletion of antioxidant enzymes.

Lung inflammation was induced in male mice using DEP for 20 days using the method of intranasal instillation. Ten days before instillation, animals were treated with different concentrations of natural treatment product *Annacardium occidentale* (cashew). The different enzymatic activities such as glutathione peroxidase (GPx), glutathione reductase (GR), glutathione S-transferase (GST) and catalase (CAT) were evaluated. Results showed that the lungs were protected by the increasing antioxidant enzyme activity of *A. occidentale* (cashew) (Carvalho et al., 2011). Similar treatment studies were performed for other air pollutant induced toxicity like 1,2-dimethylhydrazine (DMH) using natural and synthetic treatment products (Durao et al., 2011).

Treatment studies were performed in Wistar rats using synthetic products like bisdemethoxycurcumin analog (BDMC-A) on 1,2-dimethylhydrazine (DMH)-induced oxidative stress during colon carcinogenesis. The animals were given sub-cutaneous injection weekly for 5 days of DMH, DMH+BDMC-A. The group administered with only DMH showed lower lipid peroxidation with higher activities of GSH-dependent enzymes. In the other group, no tumours were observed. The Lipid peroxidation and GSH-dependent enzyme levels were also similar to that of the control group. Hence, It shows that BDMC-A offers chemoprevention against colon carcinogenesis (Devasena et al., 2005). Thus, both natural and synthetic drugs can be used for the treatment of toxicity. Treatment studies for *in vitro* and *in vivo* toxicity are underway in our laboratory.

DISCUSSION

The atmosphere is a complex dynamic natural gaseous system that is essential to support life on planet Earth. The earth's lower atmosphere or troposphere extends from the ground to a height of 15 km and is filled with breathable gases. It also contains environmental aerosols that originate from anthropogenic and biogenic activities (man-made and natural). Indoor air pollution and urban air quality are listed as two of the world's worst pollution problems in the 2008 (Blacksmith Institute World's Worst Polluted Places report). Population in India is increasing day by day and in turn increasing the urban pollution levels through increase in mass transportation vehicles. The population is mainly exposed to high air pollution concentrations, where motor vehicle emissions constitute the main source of fine and ultrafine particles, having a serious impact on our urban air quality and public health. Motor exhaust emissions is a complex mixture of gases and particulate matter (PM).

PM emitted by motor vehicles consists of fine particles, and a large fraction of these particles has an aerodynamic diameter less than 1 µm. $PM_{2.5}$ of diameter 2.5 µm can also be formed in the atmosphere as aerosols from chemical reactions that involve gases such as sulphur dioxide and nitrogen oxides. Sulfates, which are commonly generated by conversion from primary sulfur emissions, make up the largest fraction of $PM_{2.5}$ by mass. $PM_{2.5}$ can also form as a result of solidification of volatile metals salts as crystals, following cooling of hot exhaust gases from vehicles in ambient air. Gasoline fueled vehicles have lower PM emission rates than diesel-fueled vehicles. PM emissions from gasoline fueled vehicles result from unburned lubricating oil and ash-forming fuel

and oil additives. PM emitted by diesel-fueled vehicles consists of soot. Diesel exhaust particles (DEPs) are globally relevant air pollutants that exert a detrimental human health impact. However, mechanisms of damage by DEP exposure to human respiratory health and human susceptibility factors are only partially known.

CONCLUSION

Until alternative energy are fully developed and implemented, reliance on diesel fuel will increase. Acute and chronic exposure to diesel exhaust will continue to be a problem in India. This will ultimately increase the number of patient to emergency departments with pulmonary, cardiopulmonary disease, neurological disorders, and adverse perinatal events. New regulations and technology to reduce DEP emissions in vehicles by the government should be fully implemented effectively. Also, treatment using both natural and synthetic drugs against *in vitro* and *in vivo* toxicity should be explored.

ACKNOWLEDGEMENT

We would like to acknowledge the financial support provided by DST, New Delhi under the INSPIRE FELLOWSHIP SCHEME Proc No. 8946/PD6/2010.

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African Journal of Pharmacy and Pharmacology Vol. 7(7), pp. 332-346, 22 February, 2013
Available online at http://www.academicjournals.org/AJPP
DOI: 10.5897/AJPP12.014
ISSN 1996-0816 ©2013 Academic Journals

Review

Phytochemistry and mode of action of some tropical spices in the management of type-2 diabetes and hypertension

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Accepted 11 February, 2013

Spices are important food supplements and/or food products, which have been used as flavouring agents and preservatives for thousands of years in tropical Africa, Asia and other parts of the world. They are well known for their medicinal properties, and their use in traditional systems of medicine has been on record for a long time. Although, epidemiological and clinical studies have indicated that spices are important source of natural antioxidant having the digestive stimulant action, bioavailability enhancement nature, carminative attribute, antimicrobial activity, hypolipidemic property, antidiabetic influence, antioxidant capacity, anti-inflammatory ability, anticarcinogenic potential and neuroprotective effect. The present review reports the phytochemical constituents and mode of action of some tropical spices as antidiabetic and antihypertensive agents. The conclusion of this review may help in undertaking research for the development of functional foods and nutraceuticals.

Key words: Spices, type-2 diabetes, hypertension, enzymes, antioxidant, phenolics, phytochemicals.

INTRODUCTION

Spices are food supplements or food products, which have been used not only as flavoring and coloring agents, but also as food preservatives and herbs in folk medicines for thousands of years in Africa, Asia and other parts of the world (Srinivasan, 2005a). They are consumed as whole spices or ground into powder and mixed with diets containing cereals, legumes, nuts, fruits, vegetables, milk and milk products. They are also used in soup preparation in various homes and serve as ingredients in the preparation of several traditional delicacies. Spices are utilized as herbs, mainly in the form of isolates from their extracts. Spices are considered to be good contributors to the total nutrient intake of protein, carbohydrates, fats, vitamins and minerals, thereby enhancing the nutritional quality of diets (Pradeep et al., 1993). Apart from the nutrients supplied by spices, they possess many phytochemicals which are potential sources of natural antioxidant such as phenolic diterpenes, volatile oils, flavonoids, terpenoids, carotenoids, phytoestrogens, and phenolic acids (Cai et al., 2004; Suhaj, 2006; Kennedy et al., 2011).

Spice phytochemicals such as curcumin (turmeric), capsaicin (red chillies), eugenol (cloves), linalool (coriander), piperine (black pepper), zingerone (zinger) and cuminaldehyde (cumin) have been reported to inhibit lipid peroxidation (Shobana and Naidu, 2000; Oboh and Rocha, 2007). In recent times, spice antioxidants have raised considerable interest among food scientists, manufactures, and consumers because of their natural antioxidants (Lu et al., 2011). Consumers are increasingly aware of the risk posed by synthetic antioxidants due to their high volatility and instability at elevated temperatures. Therefore, focus has been shifted to the use of natural antioxidants in food preservation (Odukoya et al., 2005; Oboh and Rocha, 2007; Adefegha and Oboh, 2011a).

Food oxidation is considered a major cause of food deterioration and spoilage, causing rancidity in food (Sherwin, 1990). The resultant effect is noticed in the

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decreased nutritional quality, color, flavor, texture and safety of foods. Many spices have also been recognized to possess digestive stimulant action, carminative effect, antimicrobial activity, antioxidant capacity, antiinflammatory property, antimutagenic ability and anticarcinogenic potential (Srinivasan, 2005a). Spices contribute greatly to the daily antioxidant intake in most diets, especially in dietary cultures where spices are used as whole meal (Carlsen et al., 2010). Many spices have been shown to confer health benefits and have been proven to counteract oxidative stress *in vitro and in vivo* (Oboh et al., 2005, 2010a, 2012a; Shan et al., 2005; Wojdylo et al., 2007; Adefegha and Oboh, 2012a). They are common sources of phenolic compounds which have been reported to show superior antioxidant capacity to fruits, cereals, and nuts (Pellegrini et al., 2006; Carlsen et al., 2010). The main active components in spices are phenolic acids, flavonoids and volatile or essential oils (Shan et al., 2005; Wojdyło et al., 2007; Viuda-Martos et al., 2011; Lu et al., 2011).

In Nigeria, over 100 indigenous spices are used as important components of the “African/Nigerian dishes”, bringing original favors and desirable sensory properties to food. Essentially, “Pepper soup” is famous for its sensory, aromatic, attractive, pungency and spicy flavor resulting from the use of bastered melegueta, clove, alligator pepper, ginger, black pepper, garlic, Ethiopian pepper, chili peppers and other spices. Common spices, such as sweet basil, clove, black pepper, turmeric, chili pepper, and ginger are usually part of daily African household meals and also used as traditional African medicine. Numerous studies have reported that spices are important source of natural antioxidant, possessing digestive stimulant action, bioavailability enhancement nature, carminative attribute, antimicrobial activity, hypolipidemic property, antidiabetic influence, anti-inflammatory ability, anticarcinogenic potential and neuroprotective effect (Srinivasan et al., 2004; Shan et al., 2005; Srinivasan, 2005a; Adefegha and Oboh, 2011b).

Diabetes mellitus (DM) is one of the leading causes of global morbidity and mortality, and a major risk for cardiovascular diseases (Alderman et al., 1999). Diabetes mellitus is a metabolic disease characterized by hyperglycaemia resulting from defects in insulin secretion, insulin action, or both (World Health Organization (WHO), 1999). The control of postprandial hyperglycemia is an important strategy in the management of diabetes mellitus, especially type 2 diabetis mellitus (T2DM), and reducing chronic complications associated with the disease (Kim et al., 2000; Ali et al., 2006; Ortiz-Andrade et al., 2007). Hence, the inhibition of enzymes (α-Glucosidase and α-amylase) involved in the digestion of carbohydrates can significantly decrease the postprandial increase of blood glucose after a mixed carbohydrate diet, by delaying the process of carbohydrate hydrolysis and absorption and phenolic phytochemicals from spices and have shown promising potentials (Oboh et al., 2010b; Adefegha and Oboh, 2012a).

Hypertension or persistent high blood pressure is a common cardiovascular disease which has become a worldwide problem of epidemic proportions, affecting 15 to 20% of all adults with ailments such as arteriosclerosis, stroke, myocardial infarction and end-stage renal disease (Je et al., 2009). It is regarded as one of the long-term complications of T2DM. These two diseases (hypertension and T2DM) are interrelated metabolic disorders with persistent hypertension being the risk factors for strokes, heart attacks, heart failure and is a leading cause of chronic renal failure (Sowers and Epstein, 1995; Bakris et al., 2000). One of the therapeutic strategies towards the management of hypertension is the inhibition of angiotensin-I converting enzymes (ACE); an enzyme which play a pivotal role in rennin-angiotensin system by converting angiotensin-I to angiotensin-II (potent vasoconstrictor). However, phenolic-rich spices have reported to act as good ACE inhibitors (Ranilla et al., 2010). Therefore, the present review highlights the phytochemical constituents and mechanism of action of some tropical spices as antidiabetic and antihypertensive agents. The pictures of some spices are as shown in Figure 1

Phytochemical composition

Spices in the diet are not considered vital from the nutritional point of view, though they are widely consumed throughout the world. They are not normally included in diet surveys, nor are they suggested or recommended in what are known as balanced diets, probably because it was thought that the intake of these spices was so small that their contribution of nutrients may not be significant (Pradeep et al., 1993). As part of normal diet, plant foods are thus not only a source of nutrients and energy provider, but may confer additional role of providing health benefits beyond their basic nutritional functions (Shahidi and Naczk, 2004). Attention is being focused on identifying dietary phytochemicals which are plant secondary metabolites (array of bioactive constituents) that are capable of eliciting health enhancing effects and disease preventing abilities (Visioli and Galli, 1998).

Phytochemicals describe the chemicals present in different parts of plant organs (leaves, stems, roots, flowers, fruits and seeds). Consumption of food rich in several phytochemicals such as saponins, alkaloids, terpenes, phenylpropanoids, isoprenoids, steroids, coumarins, flavonoids, phenolic acids, lignans, contain chemicals such as flavonoids, terpenoids, lignans, sulfides, polyphenolics, carotenoids, coumarins, saponins and plant sterols with biological activities that may provide therapeutic effects (Dorman et al., 2003, 2004; Cuvelier et al., 2004; Shan et al., 2005; Ninfali et al., 2005; Adefegha and Oboh, 2012b). The presence of

Figure 1. Pictures of spices.

phytochemicals has been shown to contribute immensely to the protective potential against degenerative diseases, therapeutic effects essential to preventing diseases, and nutritional quality of food and food products (Chu et al., 2002; Oboh et al., 2010b).

Our recent study revealed the presence of flavonoid and cardiac glycoside in Ethiopian pepper [*Xylopia aethiopica* [Dun.] A. Rich (Annonaceae)], nutmeg [*Monodora myristica* (Gaertn.) Dunal (Annonaceae)], clove [*Syzygium aromaticum* [L.] Merr. et Perry (Myrtaceae)], ashanti pepper [*Piper guineense* Schumach. et Thonn (Piperaceae)], bastered melegueta [*Aframomum danielli* K. Schum (Zingiberaceae)] and alligator pepper [*Aframomum melegueta* (Rosc.) K. Schum (Zingiberaceae)], tannin in Ethiopian pepper and clove, phlobatanin and anthraquinone in clove and saponin in Ethiopian pepper, clove, ashanti pepper, bastered melegueta and alligator pepper (Adefegha and Oboh, 2012a). Other active components of spices (Figure 2) such as curcumin (turmeric), capsaicin (bird's pepper), eugenol (cloves), linalool (coriander and sweet basil), piperine (black pepper), gingerol (ginger) and allicin (garlic) have been reported to inhibit lipid peroxidation in various tissues (Lawson, 1998; Ursell, 2000; Srinivasan, 2005a; Hiyasat et al., 2009; Adefegha and Oboh, 2011b).

Polyphenols

Food contains several chemicals, many of which have specific biological activity. The chemicals also interact with each other, confounding any effort to identify bioactives. Among these bioactive components are the phenolics. Dietary phenolics are secondary metabolites which are widely present in diets rich in vegetables, fruits, legumes, cereals, nuts, and have been linked to various beneficial effects on human health, such as minimizing the risk of developing coronary heart disease, cancer, hypertension, diabetes, and inflammatory processes (Scalbert et al., 2005; Zafra-Stone et al., 2007). They are present in plants as derivatives and/or isomers of flavones, isoflavones, flavonols, catechins, and phenolic acids.

Phenolic compounds in plants can be divided into two major categories: phenolic acids and flavonoids. Phenolic acids account for about 33% of the total phenolic intake; mainly the derivatives of benzoic or cinnamic acid. Flavonoids, on the other hand account for the remaining 67% of the total phenolic intake (Scalbert and Williamson, 2000). They are regarded as the most abundant polyphenols in human diets, and are mainly divided into: anthocyanins (colourful compounds) and anthoxanthins (colorless compounds), which can be subdivided into flavones, flavans, flavonols, flavanols, isoflavones, and their glycosides (Bravo, 1998; Liu, 2004). Phenolic compounds in plants are usually found either as free or bound forms (Chu et al., 2002; Sun et al., 2002). Free soluble phenolics may be available as aglycones while the bound phenolics may be present as conjugates (glycosides and esters) or attached to the plant cell wall (Rice-Evan et al., 1996).

Hydrolysis by chemical and enzymatic means may be necessary to release the bonds (Stalikas, 2007). Most of the biological actions such as antimicrobial, hypolipidemic, antidiabetic, antilithogenic, antioxidant, antiinflammatory, antimutagenic, anticarcinogenic and neuroprotective properties observed in some spices have been attributed to the presence of phenolic compounds (Cai et al., 2004; Liu et al., 2008; Shan et al., 2005; Wojdylo et al., 2007; Muchuweti et al., 2007; Konczak et al., 2010). Shan et al. (2005) reported that spices from Labiatae, Myrtaceae and Compositae families are rich in rosmarinic acid, caffeic acid and volatile oil. In the same vein, chlorogenic acid, rutin, quercetin, and naringin were also identified as the dominant phenolic compounds in spices from the family Rutaceae (Lu et al., 2011).

In an experiment carried out on phenolic composition of three commercial herbal drugs and spices from lamiaceous species: *Thymi herba* (thyme), *Serpylli herba* (wild thyme) and *Majoranae herba* (sweet marjoram) using high performance liquid chromatography (HPLC) and high performance thin layer chromatography (HPTLC) methods, luteolin-7-O-β-glucuronide, lithospermic acid, rosmarinic acid and methyl rosmarinate, together with other known compounds, were detected and quantified. Luteolin-7-O-β-glucuronide and lithospermic acid were identified as novel wild thyme constituents, luteolin-7-O-β-glucuronide and methyl rosmarinate as novel compounds in sweet marjoram.

Methyl rosmarinate was found to be present in thyme. The amount of polyphenol investigated in herbal drugs and spices has reached 84.3 mg/g dried spices (Fecka and Turek, 2008). Previous report on phenolic composition of thyme indicated the presence of caffeic acid, rosmarinic acid, apigenin, luteolin, luteolin-7-O-β-glucuronide, luteolin-7-O-β-glucoside, 6-hydroxyluteolin glycosides, polymethoxyflavones, narirutin, eriodictyol, eriocitrin, hesperidin and taxifolin (Dapkevicius et al., 2002; Haraguchi et al., 1996; Kobayashi et al., 2003; Kosar et al., 2005; Miura et al., 2002; Watanabe et al., 2005). Wojdyło et al. (2007) also identified and quantified major phenolics by reverse-phase high-performance liquid chromatography (RP-HPLC) in thirty two (32) selected herbs and spices. The prominent phenolic acids reported were caffeic, p-coumaric, ferulic and neochlorogenic acids while the flavonoids detected were quercetin, luteolin, apigenin, kaempferol and isorhamnetin. Caffeic acid was reported as the predominant phenolic compound in sage (*Salvia officinalis*) (296 mg/100 g dry weight), thyme (*T. vulgaris*) (517 mg/100 g dry weight) and oregano (*Origanum vulgare*) (649 mg/100 g dry weight).

Luteolin (616 mg/100 g dry weight) and caffeic acid (406 mg/100 g dry weight) were also present in

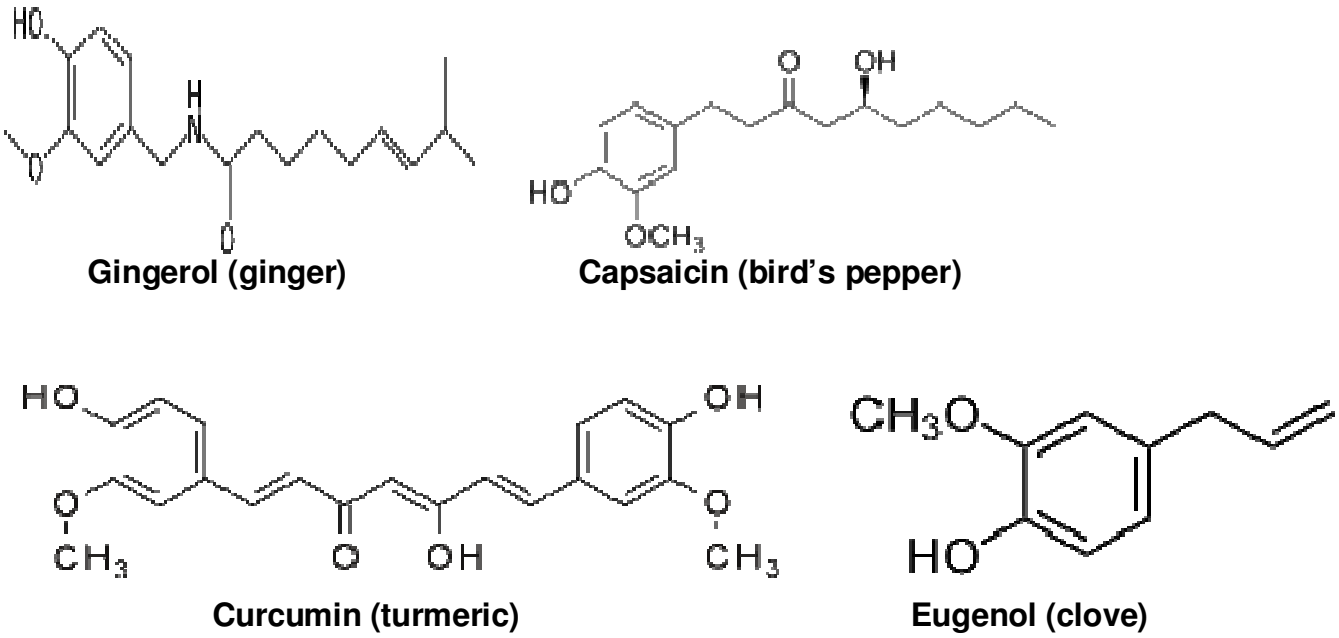

Figure 2. Active ingredients of biological relevance present in abundance in some spices.

abundance in rosemary (*Rosmarinus officinalis*). Turmeric (*Curcuma longa*) was reported to contain p-coumaric acid (5.96 mg/100 g dry weight) and ferulic acid (17.6 mg/100 g dry weight) in small quantities. A minute amount of ferulic acid was reported to be present in nutmeg (*M. fragrans*). Quercetin was the only phenolic compound detected in clove (*S. aromaticum*) (Wojdyło et al., 2007). In a similar study, the phenolic compounds of some everyday-use spice plants, such as onion, dill, parsley and celery were identified and quantitatively assessed by HPLC. Chlorogenic acid was reported as the dominant phenolic compound in celery leaves and parsley leaves, and o-hydroxycinnamic acid was dominant in dill (Stankevičius et al., 2010). Shan et al. (2005) also reported the presence of phenolic acids (rosmarinic acid, caffeoyl derivatives), phenolic diterpenes, volatile compounds (carvacrol), flavonoids (catechin) in sweet basil (*O. basilicum* L.), phenolic acids (rosmarinic acid), phenolic diterpenes (carnosic acid), volatile compounds and flavonoids in sage (*S. officinalis* L.), phenolic acids (caffeic acid, rosmarinic acid, caffeoyl derivatives), phenolic diterpenes (carnosic acid, carnosol, epirosmanol), volatile compounds (carvacrol), flavonoids in rosemary (*R. officinalis* L.), phenolic acids (gallic acid, caffeic acid, rosmarinic acid), volatile compounds (thymol), phenolic diterpenes, flavonoids in thyme (*T. vulgaris* L.), phenolic acids (gallic acid), flavonol glucosides, phenolic volatile oils (eugenol, acetyl eugenol), tannins in clove (*Eugenia caryophylata* Thunb.), phenolic volatile oils, phenolic acid (caffeic acid), flavanols (catechin) in nutmeg (*M. fragrans* Houtt) and phenolic acids (caffeic acid, p-coumaric acid, rosmarinic acid, caffeoyl derivatives), volatile compounds, (carvacrol) and flavonoids in oregano (*O. vulgare* L.).

In a similar manner, Hossain et al. (2010) reported the presence of thirty eight (38) phenolic compounds in five Lamiaceae spices: rosemary, oregano, sage, basil and thyme using Liquid chromatography coupled with electron span ionization detector and mass spectrometer (LC-ESI-MS/MS). Twenty (20), twenty six (26), twenty three (23), twenty four (24) and twenty (20) different phenolic compounds were found in rosemary, oregano, sage, basil and thyme, respectively. The structures of some of these phenolic compounds found in spices are as shown in Figure 3.

Antioxidant properties

Several oxygen-free radicals and other reactive oxygen species (ROS), which include free radicals such as superoxide anion radicals (O_2), hydroxyl radicals (OH) and non free-radical species such as hydrogen peroxide (H_2O_2) and singlet oxygen (O_2), may be formed in the human body during the normal cellular metabolism and in the food system in the course of food production and processing (Halliwell and Gutteridge, 1999; Halliwell, 2006; Oboh and Rocha, 2007). These radicals induce lipid peroxidation, thereby causing oxidative damage by oxidizing biomolecules such as proteins, lipids and DNA, leading to cell death, tissue damage and diseases such as atherosclerosis, cancer, emphysema, cirrhosis and arthritis (Kehrer, 1993). They could also result in food deterioration. On the other hand, antioxidant refers to a

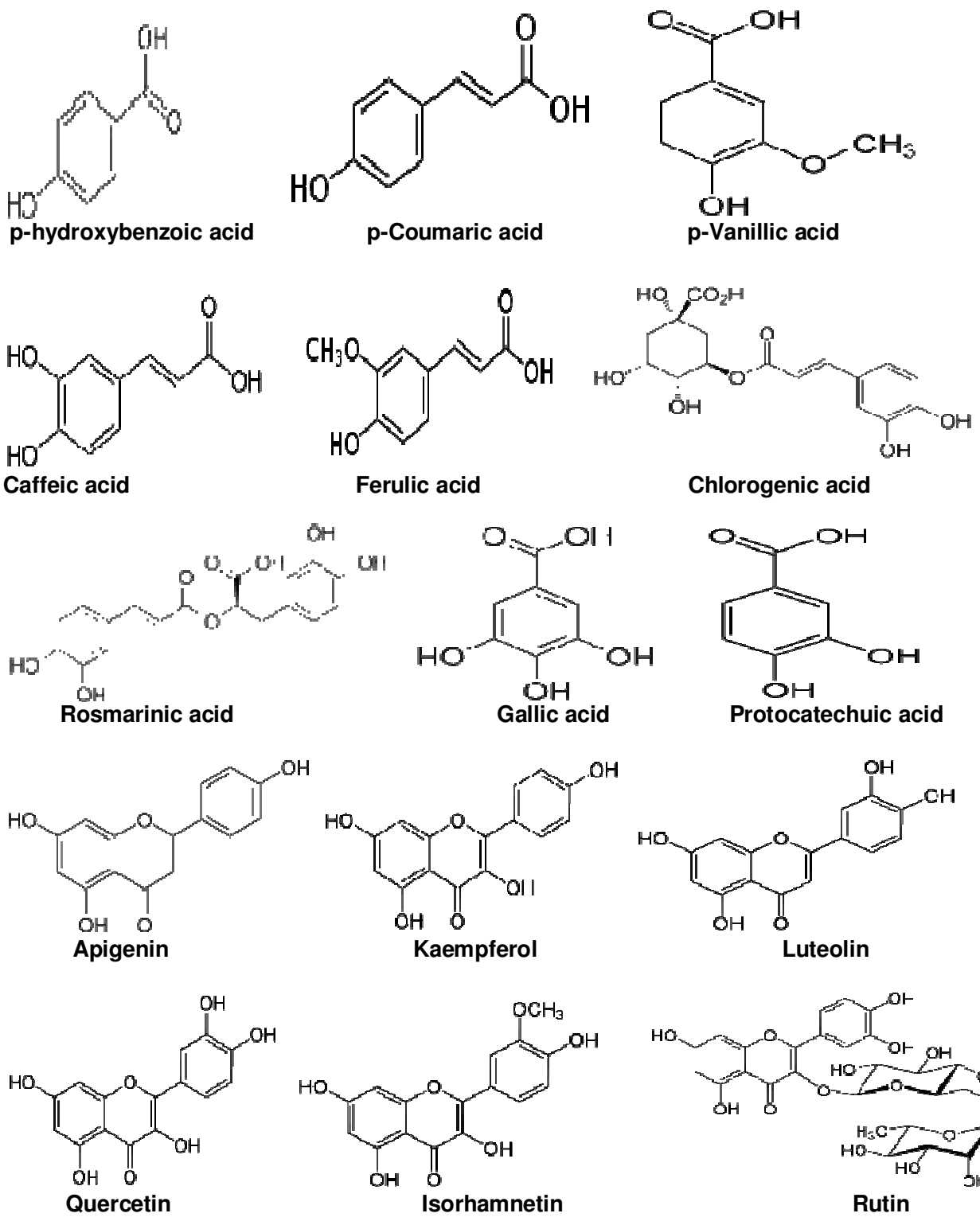

Figure 3. Structure of some phenolic constituents found in some spices.

compound that can delay or inhibit the oxidation of lipids or other molecules by inhibiting the initiation or propagation of oxidative chain reactions, and can thus prevent or repair the damage done to the body's cells by oxygen (Halliwell et al., 1995).

Antioxidants protect by contributing an electron of their own. In so doing, they neutralize free radicals and help prevent cumulative damage to body cells and tissues

(Alia et al., 2003). Much of the total antioxidant activity of plant foods is related to their phenolic content, and not only to their vitamin contents (Chu et al., 2002; Sun et al., 2002; Oboh and Rocha, 2007; Oboh et al., 2008). They exert their antioxidant activity by removing free radicals, chelating metal catalysts, activating antioxidant enzymes, reducing α-tocopherol radicals, and inhibiting oxidases (Amic et al., 2003). Antioxidant may be endogenous or exogenous (can scavenge/deactivate this reactive free radicals, turning them to harmless particles) (Chu et al., 2002). Consumption of antioxidant rich food such as spices, fruits, and vegetables could be a practical approach towards improving antioxidant status, thereby enhancing good health and preventing disease (Chu et al., 2002; Oboh and Rocha, 2007; Carlsen et al., 2010).

Since the antioxidant capacity of plant extracts from different plant foods have been attributed to their high phenolic contents, in the last years, we have assessed the total phenols and flavonoids of different plant foods (Oboh and Rocha, 2007; Adefegha and Oboh, 2011a, 2011b; Oboh et al., 2008, 2010a; 2010b; 2011; 2012a, b, c). The antioxidant properties have also been investigated using several methods: 1,1-diphenyl-2-picrylhydrazine (DPPH) radical scavenging assay (Gyamfi et al., 1999), β-carotene linoleic acid bleaching assay (Pratt, 1980), inhibition of linoleic acid peroxidation (Osawa and Namiki, 1981), ferric reducing antioxidant power (FRAP), total radical trapping antioxidant potential (TRAP) assay (Lissi et al., 1992, 1995), oxygen radical absorbance capacity (ORAC) assay (Cao et al., 1993), 15-lipoxygenase inhibition (Lyckander and Malterud, 1992), lipid peroxidation (LPO) method (Ohkawa et al., 1979), nitro blue tetrazolium (NBT) reduction assay or superoxide anion scavenging activity (Beauchamp and Fridovich, 1971), hydroxyl radical scavenging activity (Halliwell et al., 1987), hydrogen peroxide scavenging activity (Ruch et al., 1989), 2,2-azinobis-(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS) radical scavenging method (Re et al., 1999), reducing power assay (Oyaizu, 1986), ammonium thiocyanate (ATC) assay method (Masude et al., 1992) and ferric thiocyanate (FTC) method (Mitsuda et al., 1996).

In a number of studies, spices from different botanical families, such as Myristicaceae (for example nutmeg), Zingiberaceae (for example, ginger, galangal alligator pepper and bastered melegueta), Lamiaceae (for example, sweet basil, thyme, rosemary, oregano, and sage), Lauraceae (for example, cinnamon), Piperaceae (for example, black pepper, white pepper), Myrtaceae (for example, clove), Solanaceae (for example, chili pepper) and Umbelliferae (for example, Fennel, cumin, and *Angelica dahurica*), have been assessed for their antioxidant properties using some of the aforementioned assay methods (Zheng and Wang, 2001; Shan et al., 2005; Oboh et al., 2010b; Lu et al., 2011; Adefegha and Oboh, 2011b). Reports revealed that these spices possess moderate and high antioxidant activity (Shan et al., 2005; Lu et al., 2011; Adefegha and Oboh, 2011b).

Previous studies have shown that these spices are rich in phenols and flavonoids, hence their disease preventing and health promoting abilities have been attributed to the presence of these phytochemicals (Fasoyiro et al., 2006; Olonisakin et al., 2006; Wojdyło et al., 2007; Uwakwe and Nwaoguikpe, 2008; Ezekwesili et al., 2010; Doherty et al., 2010). Several studies have also correlated antioxidant capacity with total phenolic content of legumes, vegetables, pepper, spices, medicinal herbs and other plant foods (Oboh, 2006; Oboh and Rocha, 2007; Oboh et al., 2008, 2011; Oboh and Ogunruku, 2010; Adefegha and Oboh, 2011b; Oboh and Ademosun, 2011). These reports may validate the claims that phenolic compounds are responsible for most of the antioxidant effects in plants (Pietta, 2000; Chu et al., 2002; Sun et al., 2002; Cai et al., 2004; Liu et al., 2008; Shan et al., 2005; Odukoya et al., 2005; Oboh and Rocha, 2007).

Our report on the phenolic content and antioxidant properties of aqueous extract of some Nigerian spices: *M. myristica* (Africa nutmeg), *X. aethiopica* (Ethiopian pepper), *S. aromaticum* (tropical cloves), *P. guineense* (Black pepper), *A. danielli* (bastered melegueta), *A. melegueta* (alligator pepper/grains of paradise) and *Clerodendrum volubile* (Locally known as “Obenetete”)] indicated that the total phenol content of the spices range from 0.6 (*M. myristica*) to 2.28 mg gallic acid equivalents per g (mg GAE/g) (*A. melegueta*). In the same vein, *A. melegueta* (0.55 mg GAE/g) was reported to have the highest flavonoid content, followed *Clerodendrum volubible* (0.52 mg GAE/g), *P. guineense* (0.41 mg GAE/g), and *Aframomum danielli* (0.29 mg GAE/g), *Syzygium aromaticum* (0.26 mg GAE/g), *Xylopia aethiopica* (0.24 mg GAE/g) and *Monodora myristica* (0.21 mg GAE/g) (Adefegha and Oboh, 2011a).

Furthermore, the spice extracts also showed interesting antioxidant properties as typified by their ferric reducing antioxidant property, Fe^{2+}-chelating ability, inhibition of Fe^{2+}/H_2O_2-induced decomposition of deoxyribose and inhibition of Fe^{2+}-induced lipid peroxidation in rat’s brain (Adefegha and Oboh, 2011a). Shan et al. (2005) reported the phenolic contents and total antioxidant capacity of 26 spices by assessing the ability of their extracts to scavenge free radicals using the ABTS model. The ABTS radical scavenging activity of the spice extracts ranged from 0.55 (poppy) to 168.7 mmol trolox equivalents antioxidant capacity per 100 g (TEAC/100 g) dry weight (clove). Also, the total phenol content of the spices was reported to range from 0.04 to 14.38 g of gallic equivalents per 100 g (GAE/100 g) of dry weight (DW). Hence, positive correlation between the total phenol contents and the total antioxidant capacity was observed in the spices (Shan et al., 2005; Adefegha and Oboh, 2011a).

In another study where the antioxidant properties of 30 spices was assessed using FRAP, ABTS radical scavenging ability and microsomal lipid peroxidation

(MLP) assays (Hossain et al., 2008). It was reported that clove exhibited the highest ABTS radical scavenging ability, FRAP and anti-radical powers (ARP) on microsomal lipid peroxidation. Rosmarinic acid and eugenol, commonly found in clove was reported to possess higher antioxidant capacities than that of the synthetic antioxidants tested (Hossain et al., 2008). This could be an indication that natural antioxidants from spices might have more beneficial roles than the synthetic ones aside the advantage of being a safe alternative. Oxidative damage by free radicals has been implicated in the pathogenesis of vascular disease in diabetic complications, and several studies have revealed that antioxidants can attenuate these oxidative stress-induced changes in diabetes and hypertension (Ceriello, 2003; Vasdev et al., 2006). In an animal study carried out by Drobiova and his colleagues, garlic was reported to elevate serum antioxidant levels, decrease serum glucose in the garlic-treated diabetic rats and reduce systolic blood pressure in the garlic-treated hypertensive rats (Drobiova et al., 2010).

Inhibition of key enzymes linked to type-2 diabetes

Diabetes is one of the leading threats to worldwide public health and a major cause of global death (WHO, 1999). Reports mentioned that the number of people suffering from diabetes is about 171 million and this was projected to increase in geometric proportion to 366 million by 2030 (Wild et al., 2004). In all cases of diabetes, development of one or more complicated chronic diseases such as neuropathy, retinopathy, nephropathy and cardiomyopathy is common. There are two types of diabetes: type 1 and 2. Type 2 is more prevalent than type 1, and more than 90% of diabetes cases are that of the T2D.

T2D may be regarded as the second most common non communicable disorder, after hypertension in terms of public health significance (WHO, 1999). T2D is a metabolic disorder characterized by hyperglycemia, insulin resistance, insulin secretion and beta-cell dysfunction (WHO, 1999). There is growing scientific evidences that excess generation of highly reactive free radicals, largely due to hyperglycemia, cause oxidative stress, which further elevates the development and progression of diabetic complications (Johansen et al., 2005). Consequences of oxidative stress are damage to DNA, lipids, proteins, disruption in cellular homeostasis and accumulation of damaged molecules (Jakus, 2000). Oxidative stress is increased in diabetes because of multiple factors. These factors include glucose auto-oxidation, protein glycation, binding of advanced glycation end products (AGEs) to their receptors, oxidation/reduction imbalances, and reduction in antioxidant defenses can lead to increased free radical production (Penckofer et al., 2002; Rahimi et al., 2005).

The use of natural antioxidants as a complementary therapeutic approach in the management of diabetes is on the increase (Srinivasan, 2005b; Golbidi et al., 2011). Curcumin and turmeric were reported to be effective against the development of diabetic complication in rat's eyes (Suryanarayana et al., 2005), and turmeric was reported to reduce blood sugar level and modulate polyol pathway in diabetic albino rats (Arun and Nalini, 2002). In another study, curcumin, an active principle of turmeric, was reported to ameliorate diabetic nephropathy in streptozotocin-induced diabetic rats (Sharma et al., 2006). Supplementation of turmeric was also shown to attenuate proteinuria, TGF-β and IL-8 in patients with overt type 2 diabetic nephropathy and can be administered as a safe adjuvant therapy for these patients (Khajehdehi et al., 2011).

Administration of turmeric or curcumin was also reported to attenuate alloxan-induced diabetes in experimental rats (Arun and Nalini, 2002). Cinnamon is another spice that is known for its multiple health benefits. Available *in vitro*, *in vivo* and clinical evidences have indicated hypoglycaemic activity of cinnamon (Khan et al., 2003; Pham et al., 2007; Bandara et al., 2012). Although, several synthetic drugs have been developed to manage T2D but they come with their attendant side effects and are expensive. In recent times, investigations are being carried out to source natural and cheap plant foods for managing T2D and its complication through the consumption of food rich in spices, vegetables, legumes and fruits (Shim et al., 2003; Kwon et al., 2007; Ranilla et al., 2010; Oboh et al., 2010b).

Many studies have shown that inhibition of key enzymes (α- amylase and α-glucosidase) relevant to T2D could serve as therapeutic approach to the management of this disease, and some vital bioactive compounds such as polyphenols that possess interesting structure-function benefits have shown promising potentials (McCue et al., 2005; McDougall et al., 2005). α-Glucosidase and α-amylase are the key enzymes involved in the digestion of carbohydrates (McCue et al., 2005; Ali et al., 2006). α-Amylase degrades complex dietary carbohydrates to oligosaccharides and disaccharides that are ultimately converted into monosaccharides by α-glucosidase (Figure 4). Liberated glucose is then absorbed by the gut and results in postprandial hyperglycemia (Kim et al., 2000; Shim et al., 2003). The inhibition of enzymes involved in the digestion of carbohydrates can significantly decrease the postprandial increase of blood glucose after a mixed carbohydrate diet by delaying the process of carbohydrate hydrolysis and absorption (Kwon et al., 2006; Oboh et al., 2010b).

The control of postprandial hyperglycemia is an important strategy in the management of diabetes mellitus, especially T2D, and reducing chronic complications associated with the disease (Kim et al., 2000; Ali et al., 2006). This is done by retarding the absorption of glucose through the inhibition of the carbohydrate-hydrolysing enzymes α-glucosidase and α-amylase in the digestive

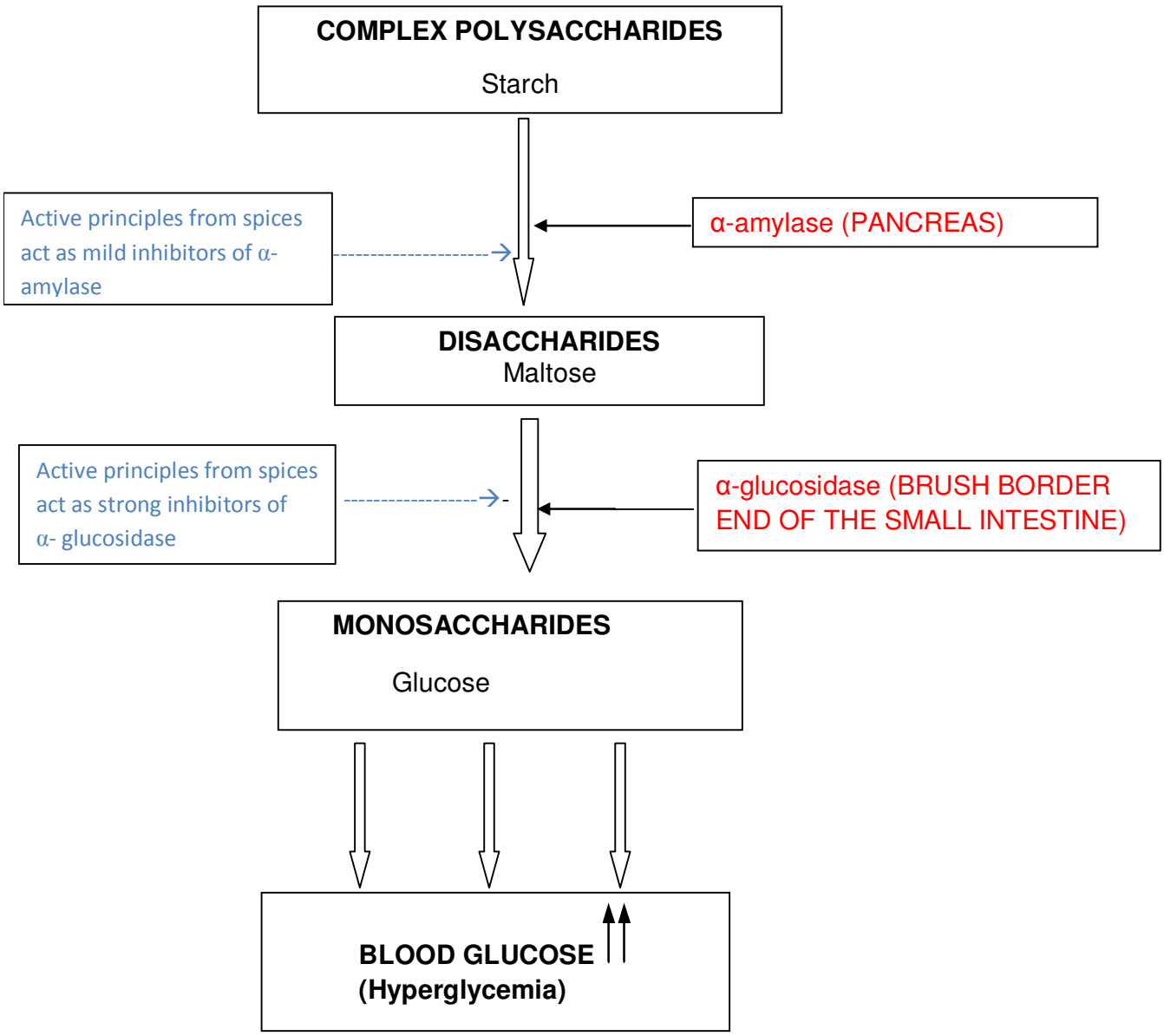

Figure 4. Schematic diagram showing the actions of α- amylase and α- glucosidase in starch digestion.

tract (Kim et al., 2000; Shim et al., 2003; Oboh et al., 2010b). Inhibitors of these enzymes could cause delay in carbohydrate digestion, prolong overall carbohydrate digestion time, causing a reduction in the rate of glucose absorption and consequently blunting the postprandial plasma glucose rise (Bhadari et al., 2008).

Several drugs such as acarbose, miglitol, voglibose, nojirimycin and 1-deoxynojirimycin have been developed and are currently in use. These drugs could act by either blocking or inhibiting these enzymes, however, they come with financial constraints and their attendant side effects, hence, alternative treatments need to be evaluated. Therefore, effective, nontoxic and cheap natural dietary inhibitors of α-amylase and α-glucosidase with little or no side effects could be potentially promising and highly desirable. Moreover, there are strong evidences that dietary factors could be involved in the regulation and prevention of T2D (Kwon et al., 2007). Several medicinal plant and plant foods have been shown to exert their antihyperglycemic activity via inhibition of carbohydrate hydrolyzing enzymes (Ortiz-Andrade et al., 2005; McDougall et al., 2005; Cheplick et al., 2007; Oboh et al., 2010b; Pinto et al., 2010; Ranilla et al., 2010).

α-Glucosidase and α-amylase inhibitors, which interfere with enzyme activity in the brush-border of the small intestine, could slow the liberation of D-glucose from oligosaccharide and disaccharides, resulting in delaying

glucose absorption and decreasing postprandial glucose levels. The toxicity of α-glucosidase and α-amylase inhibitors of natural source is much lower than that of the synthetic inhibitors. Kwon et al. (2007) reported that pepper varieties possess high antioxidant and good inhibitory profile on carbohydrate-degrading enzyme such as α-glucosidase related to glucose absorption. Green pepper and long hot pepper had little or no inhibitory effect on the α-amylase activity, which revealed their potential use with reduced side effects.

In our laboratory, two varieties of ginger were reported to show mild inhibition of α-amylase and strong inhibition of α-glucosidase, this suggest their potential use in nutritional intervention in the management or control of postprandial hyperglycemia associated with T2D. White ginger showed more promising attributes than red ginger (Oboh et al., 2010b). Nickavar and Yousefian (2009) investigated the inhibitory effects of six *Allium* spp. on α-amylase activity, and four of the selected *Allium* spp. were reported to show appreciable α-amylase inhibition. In another study, some Cameroonian spices namely, *A. daniellii, Hypodapnis zenkeri, Echinops giganteus, A. citratum, X. aethiopica* and *Scorodophloeus zenkeri* were demonstrated to have anti-amylase and anti-lipase activities, as well as good antioxidant potentials (Etoundi et al., 2010). Cazzola et al. (2011) also reported that spices such as sage, rosemary, basil, parsley, chili, garlic and onion have interesting inhibitory activities on lipid peroxidation, α-glucosidase and α-amylase.

Furthermore, our recent report also revealed the inhibitory effects of some tropical spices: *X. aethiopica* [Dun.] A. (Ethiopian pepper), *M. myristica* (Gaertn.) Dunal (nutmeg), *S. aromaticum* [L.] Merr. et Perry (clove), *P. guineense* Schumach. et Thonn (ashanti pepper), *A. danielli* K. Schum (bastered melegueta) and *A. melegueta* (Rosc.) K. Schum (alligator pepper) on α-amylase, α-glucosidase and sodium-nitroprusside (SNP)-induced lipid peroxidation in pancreas (Adefegha and Oboh, 2012a). The anti-diabetic properties of the spices were attributed to the presence of biologically active phytochemicals such as phenolic constituents of the spices. Enzyme inhibition, free radical scavenging ability and prevention of lipid peroxidation may be part of the possible mechanism of action of the spices, and this might have accounted for their usage in folklore medicine as antidiabetic gents (Oboh et al., 2010b; Adefegha and Oboh, 2012a).

In a similar manner, Ranilla et al. (2010) also reported that high phenolic and antioxidant activity-linked spices (Huacatay, *Tagetes minuta* and Guascas, *Galinsoga parviflora*), and medicinal plants (Chancapiedra, *Phyllantus niruri* L. and Zarzaparrilla, *Smilax officinalis*), and herbal teas (Yerba Mate, *Ilex paraguayensis* St-Hil) in Latin America, have strong α-glucosidase inhibitory potential with no inhibition against porcine pancreatic α-amylase *in vitro*. Furthermore, Cat's claw (*Uncaria tomentosa*), cinnamon (*Cinnamomum zeylanicum* B.), Linden tea Tilo (*Tilia platyphyllos*) and Boldo (*Peumus boldus*) were reported to strongly inhibit both α-glucosidase and α-amylase enzymes. In a related study, phenolic-rich (free and bound) extracts from clove buds also showed interesting inhibitory properties against α-glucosidase (Adefegha and Oboh, 2012b).

Ye et al. (2010) reported that some plant constituents commonly used in traditional Chinese medicine for the treatment of diabetes mellitus possess interesting inhibitory activities on α-amylase and α-glucosidase. These plant constituents are ginsenoside, puerarin, dioscin, genistein, quercetin, chlorogenic acid, taraxasterol, kaemferol, betulinic acid and paeonol from some traditional Chinese medicinal plants, and spices for treating diabetes mellitus (*Panax ginseng, P. notoginseng, Puerariae lobata, Dioscorea opposite, Astragalus membranareus, Phaseolus calcaratus Gynostemma pentaphyllum, Lonicera japonica, Paeonia lactiflora, Ophiopogon japonicas, Taraxacum mongolicum, Bupleurum chinense, Ziziphus jujuba var. spinosa*) showed strong inhibitory activities on both α-glucosidase and α-amylase (Ye et al., 2010).

Inhibition of angiotensin-I converting enzyme

Cardiovascular complications, characterized by endothelial dysfunction and accelerated atherosclerosis, are the leading cause of morbidity and mortality associated with diabetes (Johansen et al., 2005). Hypertension is one of the commonest cardiovascular diseases which have become a global epidemic affecting 15 to 20% of all adult population (Miguel et al., 2007). Hypertension means persistent increase in blood pressure (BP). According to WHO, the normal BP for an individual should be 120/80 mmHg and if it exceeds 140/90 mmHg, it is classified as 'high BP', otherwise known as hypertension. Hyperglycemia resulting from T2D may lead to hypertension; a common cardiovascular disease.

The rennin-angiotensin system (RAS) plays a key role in the regulation of blood pressure regulation in humans (Coates, 2003). Renin produces angiotensin-I, an inactive decapeptide from angiotensinogen, after which it is cleaved by angiotensin-I converting enzyme (ACE) to release a potent vasoconstrictor angiotensin-II, an octapeptide (Je et al., 2009). ACE degrades bradykinin, a vasodilator in blood vessels, and stimulates the release of aldosterone in the adrenal cortex. The ACE activity is directly linked to hypertension, as angiotensin-II is the blood pressure regulating hormone. Increased ACE activity has been linked to narrowing of lumen of blood vessels, which results in increased blood pressure (Figure 5). Therefore, inhibition of ACE activity may provide a major anti-hypertension benefits by effectively lowering hypertension (Je et al., 2009).

Synthetic ACE inhibitors such as captopril, lisinopril,

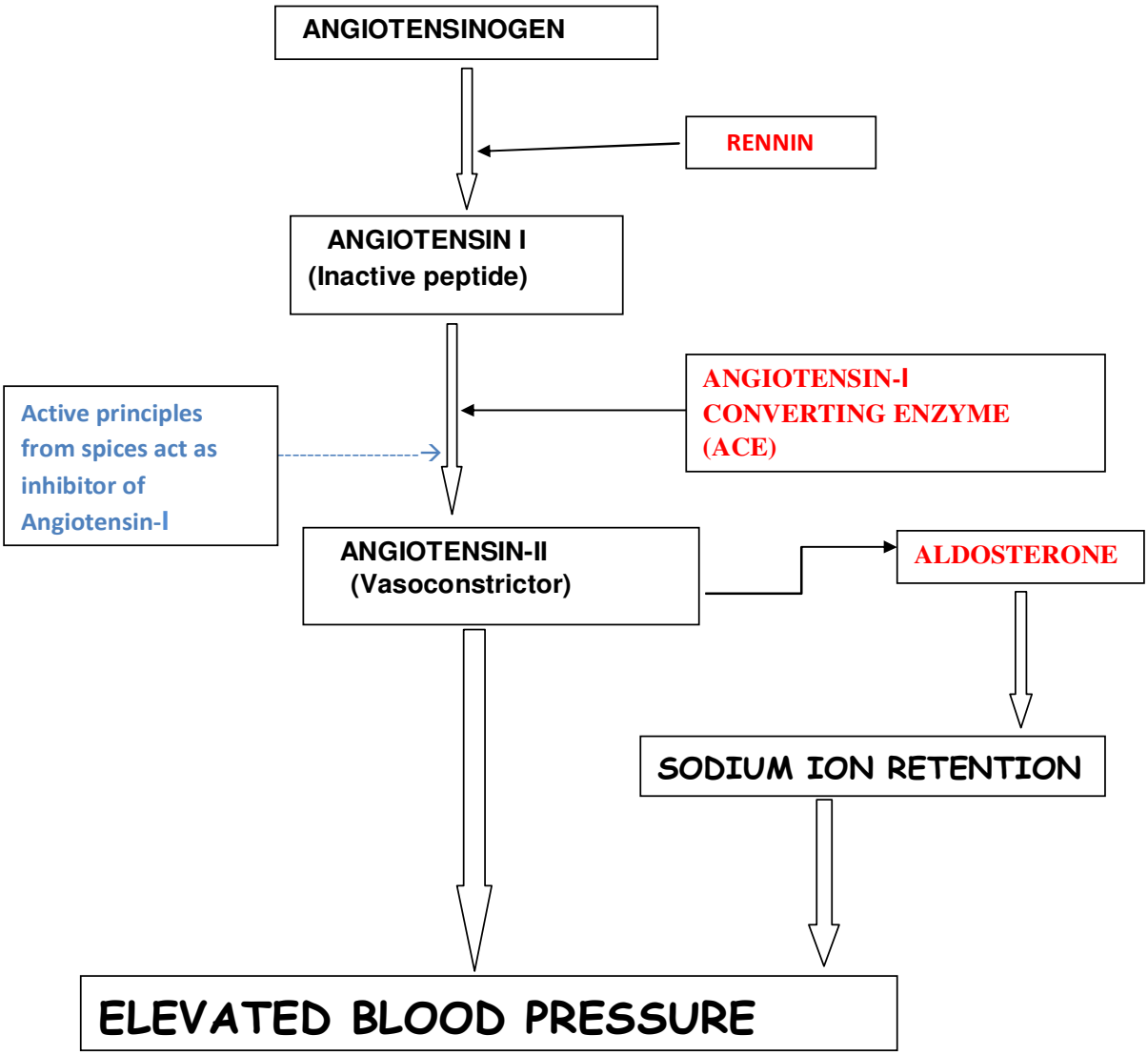

Figure 5. Schematic diagram showing the action of angiotensin-1 converting enzyme in rennin-angiotensin system.

enalapril, fosinopril and ramepril are currently in use and have shown to be very successful in controlling high blood pressure (Campos et al., 2010). They exert their antihypertensive effect by competiting for the same active site of ACE. Moreover, these drugs come with their financial constraints and their side effects such as cough, taste alterations and skin rashes. This has prompted the search for naturally-occurring ACE inhibitors, especially in flavonoids and peptides rich foods. There are indications that they are safer and lower-cost alternatives when compared to synthetic drugs (Je et al., 2009; Campos et al., 2010). Reports have shown the antihypertensive potentials and cardiovascular benefits of some medicinal plants and plant foods (Schmeda-Hirschmann et al., 1992; Hansen et al., 1995; Je et al., 2009; Pinto et al., 2009; Oboh and Ademosun, 2011; Ademiluyi and Oboh, 2012; Oboh et al., 2012c).

Ginger (*Z. officinale*) and red pepper (*Capsicum annum*) have been shown to possess high ACE inhibitory properties and could serve as dietary means of hypertension management (Ranilla et al., 2010). Clinical evidence has shown that garlic can reduce the diastolic blood pressure in hypertensive patients (McMahon and Vargas, 1993). In related study carried out to compare the cardioprotective properties of freshly crushed and processed garlic (Mukherjee et al., 2009), the authors discovered that both freshly crushed garlic and processed garlic provide cardioprotection, although the freshly crushed garlic showed a better potentials. In another clinical trial, cardamom was shown to effectively reduce blood pressure, enhance fibrinolysis and improve antioxidant status, without significantly altering blood lipids and fibrinogen levels in hypertensive patients (Verma et al., 2009).

Hypocholesteroleamic effect

Hypercholesterolemia, otherwise known as high blood cholesterol, is a major risk factor for the development of atherosclerosis and occlusive vascular disorders (Levy and Brink, 2005). WHO reported that hypercholesterolemia accounts for 18 and 56% of the world's population suffering from cerebrovascular disease and ischemic heart disease, respectively (WHO, 2002). Therapeutic life styles such as low saturated fat and cholesterol diet, weight management, and increased physical activity are vital for blood cholesterol regulation. Scientific evidences from several animal models revealed that curcumin from turmeric and capsaicin from red pepper are potent hypocholesterolaemic and hypolipidemic agents (Kempaiah and Srinivasan, 2002, 2004; Srinivasan et al., 2004).

Commercially available drugs such as statins are presently used for blood cholesterol reduction in people with or at cardiovascular risk nowadays (Endo, 2004; Kapur et al., 2008). The drug, statin, acts by the inhibition of 3-hydroxy-3-methyl-glutaryl-CoA reductase, (HMG-CoA reductase). HMG-CoA reductase is the rate limiting enzyme that catalyzes the reduction of HMG-CoA to mevalonate and provides feedback regulation of cholesterol synthesis in cells (Brown and Goldstein, 1980). Several *in vitro* and *in vivo* studies have shown HMG-CoA reductase inhibitory effects of natural plant products. In an animal model, garlic supplemented diets was reported to reduce hepatic cholesterol synthesis by the inhibition of HMG-CoA reductase (Qureshi et al., 1987). Allicin, an organosulfur compound found in spice garlic (*A. sativum*) was reported to inhibit HMG-CoA reductase in both rat hepatocytes and HepG2 cells (Gebhardt et al., 1994; Gebhardt and Beck, 1996). β-Sitosterol from black cumin seeds was found to suppress hepatic HMG-CoA reductase activity in rats (Gylling and Miettinen, 2005). Curcumin from turmeric was also reported to decrease liver enzyme in cholesterol fed rats (Murugan and Pari, 2006). Quercetin, a bioflavonoid found in the skins of red onions, significantly lowers this liver enzyme in high cholesterol fed rats (Bok et al., 2002).

CONCLUSION AND RECOMMENDATION

Consumption of spice-rich foods and their ingredients could be a more effective strategy towards the management of DM and hypertension. The advantages of spice-rich food and spice antioxidant could be associated with high compliance and absence of side effects. Spice rich foods and spice antioxidant may exert their actions by possible inhibition of key enzymes linked to TY2DM (α- amylase and α-glucosidase), hypertension (ACE) and hypercholesterolaemia (HMG-CoA reductase). Spice phenolics may also serve as potential hurdles to counter the complications of diabetes arising from oxidative dysfunction. Spices have been shown to possess good nutrient benefits with low calories, possess good inhibitory profiles on carbohydrate-modulating enzymes, ACE and HMG-CoA reductase, which correlates to their total phenolic contents, phenolic profile and antioxidant properties. This review points out the potential of spices, especially from the tropics for both T2D-linked hyperglycemia, hypertension and hypercholesterolaemia management. It also projects spice based diets as an effective dietary strategies for controlling early stages of postprandial hyperglycemia and associated hypertension. Overall, this review provides the biochemical rationale for further animal and clinical studies.

Due to the increased incidence and prevalence of several degenerative diseases such as diabetes, cardiovascular diseases including hypertension, cancer and neurodegenerative diseases including Alzheimer's diseases, concomitant drug resistance actions to these diseases and their attendant side effects, we therefore recommend an alternative dietary therapy via increased consumption of whole spice meal and spice-rich food. In a nutshell, we say "Spice up your life". This assertion supports what the great philosopher Hippocrates said about food "you are what you eat".

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African Journal of Pharmacy and Pharmacology Vol. 7(7), pp. 347-355, 22 February, 2013
Available online at http://www.academicjournals.org/AJPP
DOI: 10.5897/AJPP12.488
ISSN 1996-0816 ©2013 Academic Journals

Full Length Research Paper

Design, characterization and evaluation of PEGylated-mucin for oral delivery of metformin hydrochloride

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Accepted 13 November, 2012

The study was undertaken to formulate and evaluate PEGylated-mucin matrices-based solid microparticles for oral administration of metformin hydrochloride (MTH). PEGylated-mucin matrices formulated with PEG-2000 and Mucin were used to prepare metformin-loaded PEGylated-mucin using solvent interaction method. Characterizations based on size and morphology, zeta potential and polydispersity index, loading and encapsulation efficiency (EE%) were carried out on the PEGylated matrices. *In vitro* release of metformin from the preparation was performed in phosphate buffer while *in vivo* release as a function of the antidiabetes effects were conducted in alloxan induced diabetes rats. Maximum and minimum EE% of 81.0 and 44.0% were obtained for matrices formed with PEG-Mucin ratio of 3:1(D) and 0:1(B), respectively. Irregular and rough matrices of size range 58.80 ± 0.21 µm to 124.1 ± 0.1 µm were produced. The release of MTH in phosphate buffer varied widely with the PEG and Mucin contents. Moreover, significant (p<0.005) amount of MTH was released *in vivo* from the matrices as demonstrated in the basal glucose reduction than the positive control. These results demonstrated that PEGylated matrices would likely to offer a reliable means of delivering metformin orally.

Key words*:* PEG–Mucin, diabetics, metformin, bioactivity.

INTRODUCTION

In recent time, formulation scientists have doubled their effort towards the development of novel and oral controlled release drug delivery systems to provide a long-term therapeutic concentration of drugs following a single dose. Many controlled release drug delivery systems are based on polymers and their conjugates (Rouge et al., 2006). But, there are several physiological difficulties, which include restraining/localizing the drug delivery system within the regions of the gastrointestinal tract and the high variable nature of gastric emptying process (Rouge et al., 2006). The major absorption zone, stomach or upper part of intestine can contribute to incomplete drug release from the drug delivery system leading to diminished efficacy of the administered dose. Therefore, localizing the drug delivery in a specific region of the gastrointestinal tract due to its mucoadhesiveness increases the intimacy and duration of contact between the drug containing polymer and the mucous surface. Such a drug delivery system offers numerous advantages, especially for drugs exhibiting an absorption window or for drugs with a stability problem in the stomach. Overall, the intimate and prolonged contact of the drug delivery system with the absorbing membrane has the potential to maximize the rate of drug absorption (Chowdary and Srinivasa, 2003; Pothal et al., 2004). These considerations have lead to the development of oral controlled release microcapsules /microspheres possessing mucoadhessive properties (Pothal et al., 2004).

Metformin is an oral anti-hyperglycemic agent, which shows incomplete absorption from the gastrointestinal tract and the absolute bioavailability of 50 to 60% with relatively short plasma half-life of 1.5 to 4.5 h. Oral absorption of metformin is confined to the upper part of

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the intestine (Indian Pharmacopoeia, 1996). Therefore, the low bioavailability can be ascribed to a comparatively high pre-systemic clearance that takes place after administration. More importantly, are the gastrointestinal side effects such as abdominal discomfort, nausea, and diarrhea associated with metformin intake and the need for repeated administration to maintain an effective plasma concentration, that at times decreases patient compliance (Murugesh, 2003). It may therefore be more desirable to deliver the drug in a sustained release dosage form. However, contributions by pharmaceutical and biomaterials scientists have led to the development of novel carriers to be used as innovative drug-delivery systems. The performance of these systems was improved by synthesizing polymers that have desirable chemical, physical and biological properties, and favourable interactions with biological materials. These advancements have started to unlock ways around the problems that are encountered with oral drug delivery (Davis, 2006).

Among these measures, PEGylation is one of the most popular methods. PEG (polyethylene glycol) is a non-toxic and non-irritant hydrophilic polymer (Zabaleta et al., 2007). PEGylation of proteins, drugs, liposomes, and nanoparticles has been proven to be an effective approach for extending circulation in the blood stream, owing to the steric hindrance of the PEG chains (Gao et al., 2007; Hong et al., 2004). Similarly, PEGylated polymers may improve the stability of the drug delivery system in the blood by preventing protein absorption and uptake by reticuloendothelial systems (RES) (Tsutsumi et al., 1999; Shibata et al., 2004). Mucins are high molecular weight glycosylated proteins, believed to be the major structure-forming component of mucus (Bloofield, 1983) and responsible for the cohesive and visco-elastic nature of mucus gel (Mortazavi et al., 1992). Pure mucin is the glycoprotein part of the mucus devoid of water, free proteins, minerals and lipids (Adikwu et al., 2005). Apart from acting as protectants and lubricants, mucins are known to be the substrate on which muco-adhesive polymers attach, thus the interest in hybridizing them with other polymers (Builders, 2008).

PEGylation-based polymers have been extensively used for mucoadhesive applications because they exhibit high adhesive bond strengths on contact with tissues (Lowman et al., 1997). This increases residence time and drug bioavailability in most cases. Researchers believe that, in such systems, the free PEG chains that were grafted onto the PEGylation act as mucoadhesive anchors, causing an increase in mucoadhesion and making these carriers very promising as systems for drug delivery (Lowman et al., 1997). Scientists race to discover better ways to improve the adhesive properties of polymers such as using linear PEG as an adhesion promoter, neutralizing ionic polymers and developing polymer combinations to provide better drug delivery system (Momoh et al., 2010; Nakamura et al., 2004).

The primary objective of this study was to develop PEGylated-mucin based microparticulate delivery system that would enhance the sustained release of metformin hydrochloride which will lead to improved bioavailability of the drug.

MATERIALS AND METHODS

The following materials were used: polyethylene glycol 2000 (Cary Roth, Germany), monobasic potassium phosphate, sodium hydroxide and concentrated hydrochloric acid (BDH, England), distilled water (Lion water, University of Nigeria Nsukka). Metformin hydrochloride pure sample was obtained as a gift from Farmex Meyer Pharma Ltd. (Ikeja, Lagos State, Nigeria). Other reagents were of analytical grade and used without further purification.

Extraction of snail mucin

After procurement, shells of the giant African land snails were knocked open at the apex and a spirally coiled rod inserted to remove the fleshy body from where the excretory parts were removed. The fleshy parts were then placed in 250 ml of water and washed several times until the slimy mucin was completely washed off. These washings were pooled together in an aluminium bucket, precipitated with chilled acetone and then dried by lyophilization. Briefly, the mucin mucilage was rapidly frozen in an ethanolic bath at -25°C for 25 min and then placed in a vacuum chamber of the lyophilizer (Labconco Freezone apparatus, USA). The lyophilisation was carried out at -25°C under vacuum (1.20 to 3.0 mbar) for 24 h. The greyish-brown lyophilized flakes of the snail mucin were collected and pulverized into fine powder using a mortar and pestle and stored in an airtight container until used.

Preparation of unloaded PEGylated-mucin

Unloaded PEGylated-mucin matrices were prepared by solvent method. Briefly, 5 g of PEG and Mucin were dissolved separately in 30 ml of highly purified water with a magnetic stirring set up (300 rpm) in a 100 ml beaker until a clear solution was obtained. After 4 h, the mucin mix was then dispersed in the PEG solution using magnetic stirring (300 rpm) and allowed to stand undisturbed for 24 h. A 500 ml volume of chilled acetone maintained at -20°C was then slowly added into 250 ml beaker containing the pegylate with constant stirring at a speed of 100 rpm, for 30 min, in an ice to regulate the temperature (owing to the nature of mucin). The PEGylated-mucin samples formed were collected by filtration through a milliport filter 0.22 µm. Detail of the ratios are shown in Table 1.

Loading of metformin into the pegylated-matrices

Metformin was loaded into the various matrices by the diffusion loading method. A 2.0 g-quantity of the pegylated-mucin matrices (Table 1) was placed in a 50 ml beaker and 500 mg quantities of the metformin previously dissolved in 5 ml of highly purified water, added. This was allowed to stand for 3 h in an ice jar. The resulting matrices after hydration were freeze dried and then sieved using a standard sieve of size 100 µm. The matrix was stored in an air tight container and maintained at 10°C until used for further study.

Table 1. Ratios of mucin and PEG used for preparing unloaded microparticles.

Batch code	PEG	Mucin
A	1	1
B	0	1
C	1	2
D	3	1
E	1	3

Characterisation of unloaded PEGylated-mucin matrices

Morphology, particle size and zeta potential analysis

The morphology of the PEGylated-mucin matrix was obtained by field emission scanning electron microscopy (SEM) (JEOL JSM-6500F, Tokyo, Japan) under an accelerated voltage of 4 KV and a working distance of 6 mm. A drop of sample dispersion was spread onto a metal slab and the excess droplets removed with a filter paper. The samples were then coated in a cathode evaporator with a fine carbon layer and observed by scanning electron microscope (SEM). The particle size of the PEGylated-mucin matrix was determined by photon correlation spectroscopy using a Zetasizer nano (ZEN 3600, Malvern, UK). Size distribution analysis was performed according to an earlier report (Nakamura et al., 2004). Briefly, the samples were diluted with highly purified water and the electrophoretic mobility determined at 25°C and a light-scattering detection angle of 90°C. The electrophoretic mobility values obtained were used to calculate the zeta potentials employing a computer software (DTS Version 4.1 Malvern, UK).

The mean particle size and polydispersity index were determined. The zeta potential was similarly determined using a Zetasizer by phase analysis light scattering.

Differential scanning calorimetry (DSC)

The degree of crystallinity and changes in heat capacity of the PEGylated-mucin was determined using a calorimeter (DSC 220°C) connected to a disc station (Mettler Toledo, Greifensee, Switzerland). Briefly, about 4 to 5 mg of the PEGylated-mucin was weighed into an aluminium pan, sealed hermetically, and the thermal behavior determined in the range of 20 to 220°C at a heating rate of 5°C/ min and then cooled back to 20°C. This analysis was carried out in all the samples of the PEGylated-mucin. The baselines were determined using an empty pan, and all the thermograms were baseline-corrected. Transition temperatures were determined from the endothermic peak minima while transition enthalpies (where possible) were obtained by integration of the endothermic transitions. The results were expressed as the mean of three independent measurements.

Determination of drug loading (DL) and encapsulation efficiency (EE)

About 50 mg of metformin-loaded microparticles were dissolved in 10 ml of phosphate buffer (pH 7.4) and added into a microconcentrator (5000 MWCO Viva science, Germany). This was centrifuged (TDL-4 B. Bran Scientific and Instru. Co. England) at 1500 rpm for 120 min. The supernatants were adequately analyzed with a spectrophotometer (Jenway 60172, Germany) at 242 nm. The amount of drug encapsulated in the microparticles was calculated reference to a standard Beer's plot to obtain the % encapsulation efficiency (EE) using the formula:

$$DL\ (EE) = \frac{\text{Total quantity of the drug- quantity in supernatant}}{\text{Total quantity of the carrier}} \times 100 \quad (1)$$

***In vitro* drug release**

The *in vitro* release profiles of the metformin-loaded microparticles were determined. Approximately, a 100 mg quantity of the metformin-loaded microparticles was filled into hard gelatin capsules. Each capsule was then placed in a 250 ml beaker containing 200 ml of phosphate buffer solution PBS (pH 7.4). Agitation of the fluid system (100 rpm) was done with a magnetic stirrer. At determined intervals, 1 ml samples were withdrawn and fresh PBS added. The withdrawn samples were filtered through a 0.22 µm Millipore® filter and analyzed for metformin content using a spectrophotometer (Shimadzu, A160, Japan) at 242 nm.

Effect of the preparation on basal blood glucose level

Experimental rats

Wistar strain albino rats of both sexes weighing between 160 to 200 g were bred in the Department of Biochemistry, University of Nigeria, Nsukka. The animals were housed in standard environmental conditions and kept at body temperature of 37°C using warming lamps. All the animals were fasted for 12 h, but were allowed free access to water, before commencement of the experiments.

Induction of experimental diabetes

Rats of either sex weighing 160 to 200 g were fasted for 18 h before the induction of diabetes. Diabetes was induced with single intraperitoneal injection of 0.5 ml alloxan monohydrate dissolved in normal saline (0.9% NaCl) at a dose of 200 mg/kg body weight. Alloxan is known to inactivate the pancreatic cells without reducing exocrine functions. Blood glucose levels were monitored daily for 5 to 7 days using a Glucometer (Accu-Check, Switzerland) for all the rats until the induction and stabilization of diabetic state. After a week of the alloxan treatment, rats with frequent urination, loss of weight, and blood glucose levels higher than 200 mg/dl were selected and randomly divided into six groups. Before the administration of testing agent, animals were fasted overnight with free access to water. Animal ethical procedures were strictly followed in accordance with the requirements of the Ethical Committee, Faculty of Pharmaceutical Sciences, University of Nigeria, Nsukka.

Administration of metformin loaded-PEGylated-mucin matrices

The 24 diabetic rats were randomly divided into six groups of four with each group housed in a separate cage. The different formulations of the metformin-loaded microparticles were filled into hard gelatin capsules, with each capsule containing microparticles equivalent to metformin dose of 10 mg/kg body weight for each animal. The capsules were administered orally to the animals according to their weight as follows: rats in group one received formulation labeled as batch A, the second group received formulation

D, rats in group three received formulation E, while groups four, five and six received marketed sample (M-S), metformin HCl dispersed in distilled (D-W) and distilled water (W), respectively. In order to evaluate the effect of formulations on glycemia, groups four and five were used as positive control and group six served as negative control. In each case, the formulation was administered using gastric nasal tube. Blood samples were taken from the tail of the rats at predetermined time intervals of up to 24 h after drug administration and examined for the basal blood glucose level using a glucometer (Accu-Check, Switzerland).

The post-dose levels of the blood glucose were expressed as a percentage of the pre-dose level. The percent basal blood glucose concentration was plotted against time for the various groups.

$$\% \text{ Glycaemic change} = \frac{InitialConc - FinalConc}{InitialConc} \times 100 \quad (2)$$

Data analysis

All experiments were performed in replicates (n = 3) for validity of statistical analysis. Results were expressed as mean ± SD. ANOVA and Student's t-tests were performed on the data sets generated using Origin for Windows. Differences were considered significant for p values < 0.05.

RESULTS AND DISCUSSION

Morphology, particle size and zeta potential

Preliminary thermal analysis carried out on PEGylated-mucin matrices shows that there was an interaction between the mucin and PEG-2000 (Momoh, 2010). Hence, this method was employed to pegylate mucin and PEG-2000 which was used in this work. The SEM photomicrographs of the unloaded PEGylated-mucin matrices of batch 1 to 5 obtained by scanning electron microscopy are shown in Figures 1a to e. The micrographs (batch A to C) show the formation of non spherical microparticles with rough porous surfaces and irregular shape. Batches D and E, though similar in shape to batches A to C are characterized by larger and less spherical particles with denser and rougher surfaces. This was further confirmed by particle size analysis, which showed that size distributions vary from 72.0 to 124.0 µm. There was a clear distribution of both small and large particles with little agglomeration. Study has shown that the morphology of polymers used in drug delivery plays an important role in controlling the release characteristics of formulated drugs (Jain et al., 1998). This is because the size and number of pores determine the rate and extent of drug release from the polymer matrices. Generally, the shape and size of the particles are affected by the combination ratios of PEG and mucin. The mean particle size range of the unloaded PEGylated-mucin matrix as determined by photon correlation spectroscopy Zetasizer nano was found to be 71.0 to 124 µm (Table 2). There were significant variations in their particles sizes and the narrowest and densest range of particle dispersion are found in preparation C and D, respectively. The PDI values of the various preparations A to E were 0.745, 0.885, 1.000, 1.000 and 1.000, respectively. Generally, PDI is a measure of the distribution of molecular mass in a given polymer sample.

Values in the range of 0.95 to 1.00 are considered to be the best for particles intended for oral drug delivery (Jain et al., 1998). Values are usually greater than 1, but as the polymer chains approach uniform chain length, the value becomes 1.000 which is considered as the best as seen in preparations C to E, and is mucin concentration dependent, although preparation D proved otherwise. The size distribution patterns of the preparations show a unimodal distribution. On the other hand, zeta potential values varied between -18.7 to -23.2 mV as shown in Table 2. The significance of zeta potential is that its value can be related to the stability of colloidal dispersions (Bell, 2004). For molecules and particles that are small enough, a high zeta potential will confer stability, because the solution or dispersion will resist aggregation (Bell and Rodriguez, 2004). When the potential is low, attraction exceeds repulsion and the dispersion will break and flocculate. Those with high zeta potential (negative or positive) are, therefore, electrically stabilized while colloids with low values tend to coagulate or flocculate (Hackley et al., 2002). Zeta potential is widely used for quantification of the magnitude of the electrical charge at the double layer. Although, it is not equal to the stern potential or electric surface potential in the double layer, it is often the only available path for characterization of double-layer properties and can be calculated using theoretical models and an experimentally-determined data.

When the results of electrophorectic mobility were compared, PEG: mucin (0:1) matrices B and PEG: mucin (3:1) D had lower mobility values of -1.479 and -1.521, respectively as compared to those of PEG: mucin (1:1) of matrices A, PEG: mucin (1:2) C and PEG: mucin (1:3) E, which were -1.870, -1.810 and -1.823 mS/cm, respectively (Table 2). The zeta potentials and electrophoretic mobilities determined were mucin concentration-dependent. The magnitude of zeta potential gives an indication of the potential stability of a dispersion system. Large negative or large positive zeta potential is required for dispersion stability. The general dividing line between stable and unstable suspension is generally taken as either +30 or -30 mV (Kosmulski and Rosenholm, 2004). The relatively high negative zeta potentials of the produced mucin PEGylation would contribute to stability of the dispersion. From the data, it is evident that all the formulations are stable in solid form

Table 2. Characterization of the PEGylated- mucin matrices.

Batch	PEG	Mucin	Z-ave	PDI	ZP	Mob	LD	% EE	Particle size
A	1	1	412	0.745	-23.0	-1.870	34±0.4	49±0.2	71.1±0.41
B	0	1	1283	0.885	-18.7	-1.479	23±0.1	44±0.1	114.2±1.4
C	1	0	4487	1.000	- 23.1	- 1.810	30±0.2	63±0.4	58.8±0.21
D	3	1	569	1.000	- 19.4	-1.521	56±0.6	81±0.0	124.1±0.1
E	1	3	2863	1.000	- 23.2	-1.823	52±0.1	74±0.3	76.6±0.11

PDI = Polydispersibility index, ZP = zeta potential, Mob = mobility, Z-ave = average particle diameter, LD = Loading dose, EE = encapsulation efficiency.

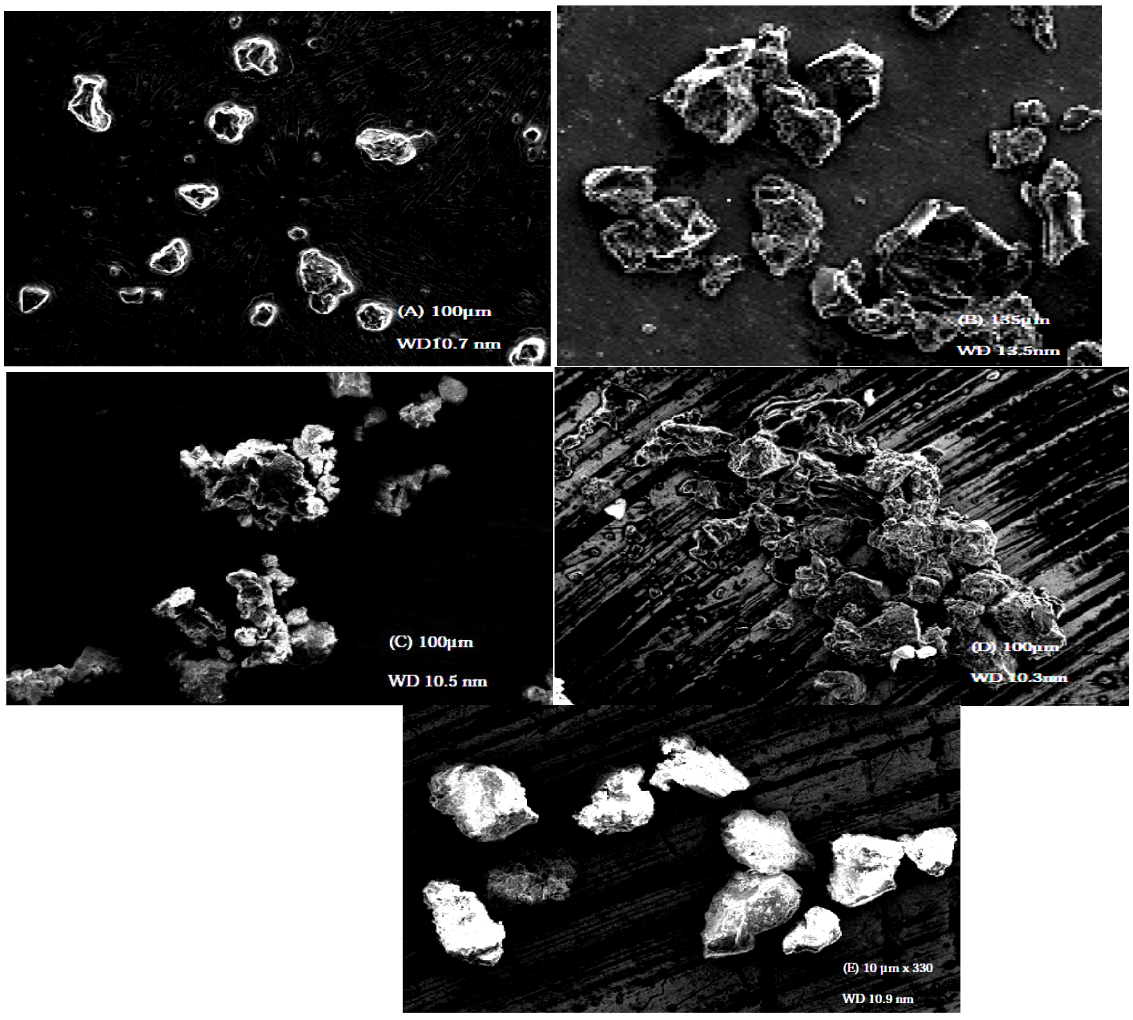

Figure 1. Scanning electron microscopy of unloaded PEGylated-mucin matrices (A-E).

but very unstable in a colloidal state.

The DSC study on the PEGylated samples

The melting behaviours and consequently, the likely release potentials of the PEGylated products were investigated by DSC. Low enthalpy transitions were obtained for some of the preparations as presented in Figures 2a to e. The thermotropic phase behaviour of a non-ionic amphiphilic polymer PEG is highly affected by the presence of mucin, and the related thermodynamic

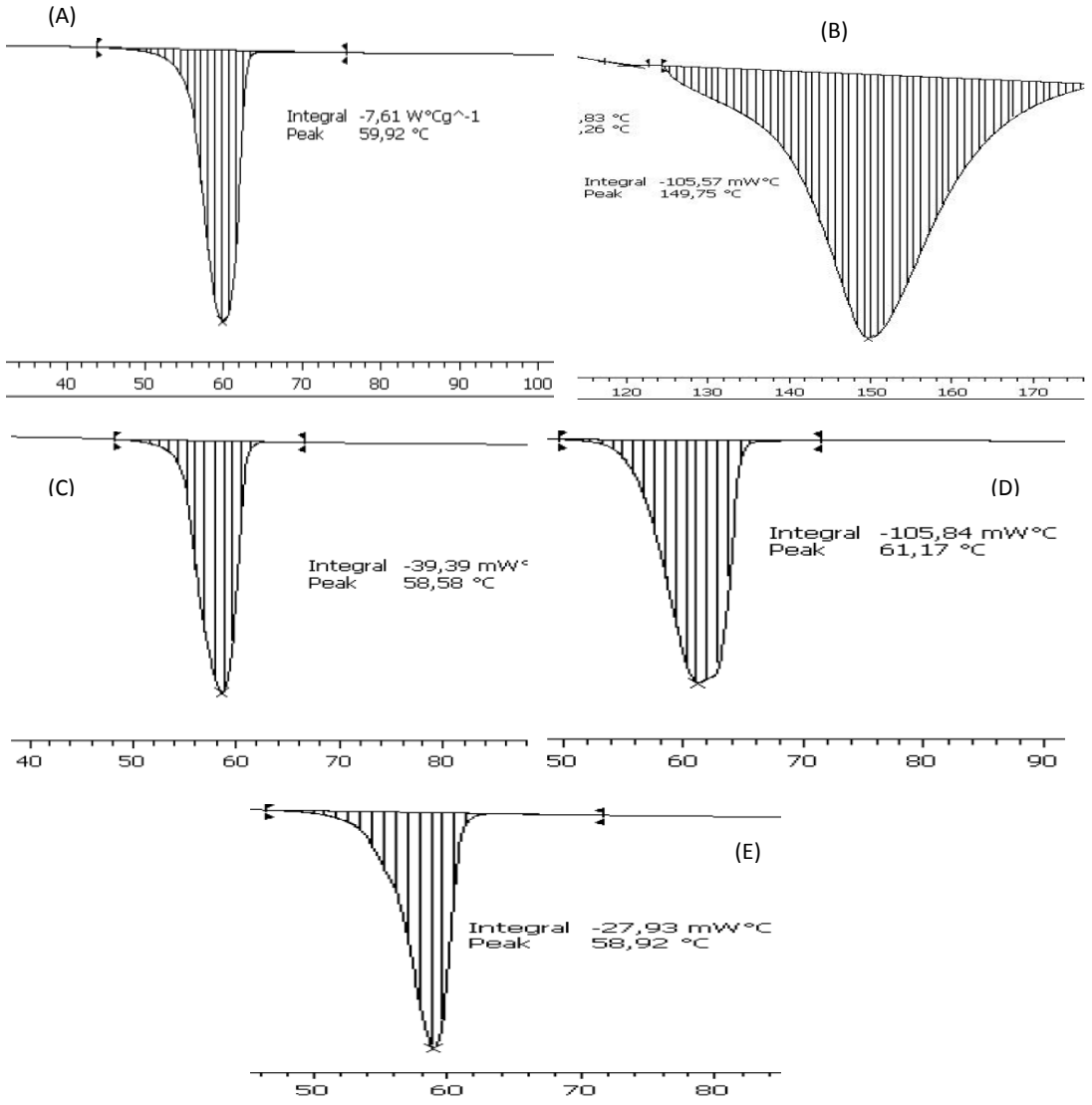

Figure 2. DSC thermographs of the unloaded PEGylated-mucin of (1:1) (a), (0:1) (b), (1:0) (c), (3:1) (d) and (1:3) (e).

variables (melting temperature and enthalpy changes) depend on the nature of the interaction between the two components (mucin and PEG). A very broad peak and two shallow endothermic peaks were detected for the pure mucin prepared without PEG with an enthalpy at low temperature of 70°C and the two enthalpy peaks appeared very shallow at about 250 and 325°C (Figures 2a to e). Batches A, C, D and E showing close peaks at a low enthalpy of 70°C, with two additional transition peaks at 350 and 425°C of PEG prepared with mucin were obtained for each formulation A and C, respectively. The high enthalpy suggests high crystallinity and possible release of the drug that may be entrapped into the preparation when used to deliver drug over time.

The compressed results of the thermograms obtained for preparation containing PEG and mucin were drastically reduced, while some peaks were absent, indicating the interaction between the two components produced a single component.

Encapsulation and loading efficiency

Table 2 shows the metformin encapsulation loading efficiency of the PEGylated-mucin matrices formulated with the various ratios of PEG and mucin. The formulation was freeze dried to minimize loss of mucin integrity due to heat which will eventually affect the drug availability. High drug loadings were observed in Batches D to E. Metformin loading generally increased with

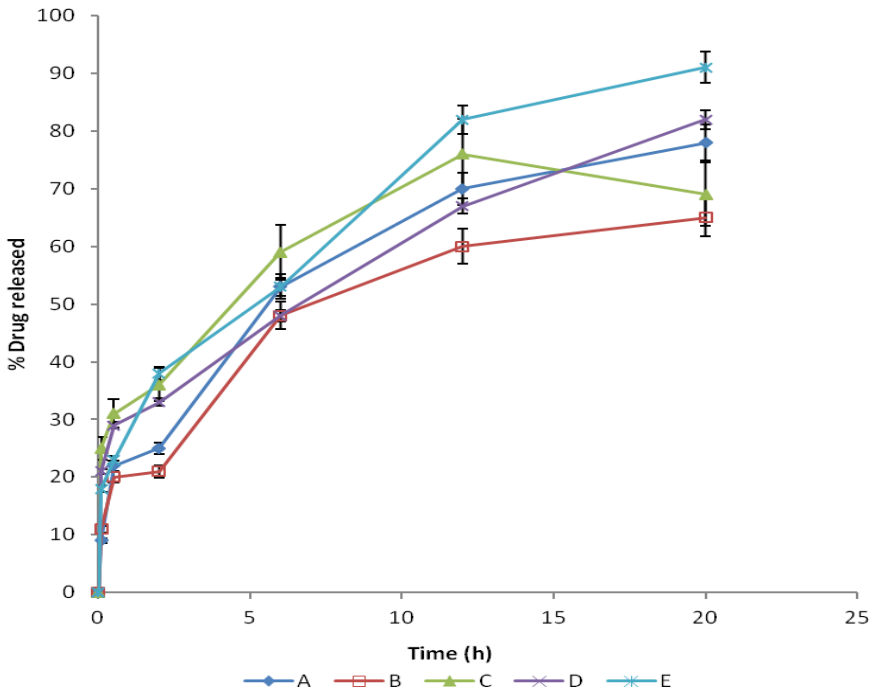

Figure 3. *In vitro* release profile of metformin from PEGylated-mucin matrix [A = (1:1) of PEG-Mucin, B = (0:1) of PEG-Mucin, C = (1:) of PEG-Mucin, D = (3:1) of PEG-Mucin and E = (1:3) of PEG-Mucin]. Each of the batches contained 500 mg of metformin HCl.

of the drug was released from the capsule within the first 1.5 h, followed by a mass release within 20 h the study lasted. However, the initial release (burst effect) may be an indication of the unencapsulated metformin which possibly only adsorbed to the surface of the PEGylated material, hence, the ease of the drug to get into the release medium at a fast rate. The second release observed here could be attributed to slow migration of the drug that get into the core of the PEGylated-mucin to the surface and finally into the medium. However, the release rates of metformin were prolonged in the case of preparations C and D than observed in B and C, the loaded non-PEGylated polymers (PEG or Mucin), although there was no significant difference in the release rate of drug from these preparations.

Metformin is a known hydrophilic drug and belongs to the class III compounds according to the BCS. Therefore, the release rate of metformin from the PEGylated-mucin matrix systems was dependent on the mucin more than the PEG used.

Result of glucose lowering effect

The hyperglycaemic state of the rats after the administration of alloxan was monitored for 24 h. This was to ensure that there was a persistent raise in blood glucose level before the commencement of the study. Other signs such as polyurea, polyphagia and polydypsia (3Ps) were obviously present. Alloxan became the first diabetogenic chemical agent when Dunn and Letchie accidentally produced islet-cell necrosis in rabbits while researching the nephrotoxicity of uric acid derivatives. Alloxan is a specific toxin that inactivates the pancreatic β cells, provoking a state of primary deficiency of insulin without affecting other islet types (Dunn et al., 1943). Hence, alloxan was selected to induce diabetes in the present study. Based on the release study result, batch A, D and E were selected for *in vivo* evaluation on animal model. Results in Figure 4 indicate a good hypoglycemic effect with significant glycemia decrease for all the formulations. The mean blood glucose baseline (initial glucose level) value was taken as the 100% level and all other blood glucose level/time data were calculated as a percentage of the baseline. Rats in group (W) that received distilled water maintained a very high blood glucose level. No apparent hypoglycaemic response was observed in this group that served as negative control.

Orally administered metformin dispersed in distilled (labelled DW) water resulted in a slight fall in the blood glucose level within 0.5 h of administration, and maintaining the temperature up to 6 h. The percentage blood glucose reduction for the formulations administered was significantly ($p < 0.05$) higher than the marketed

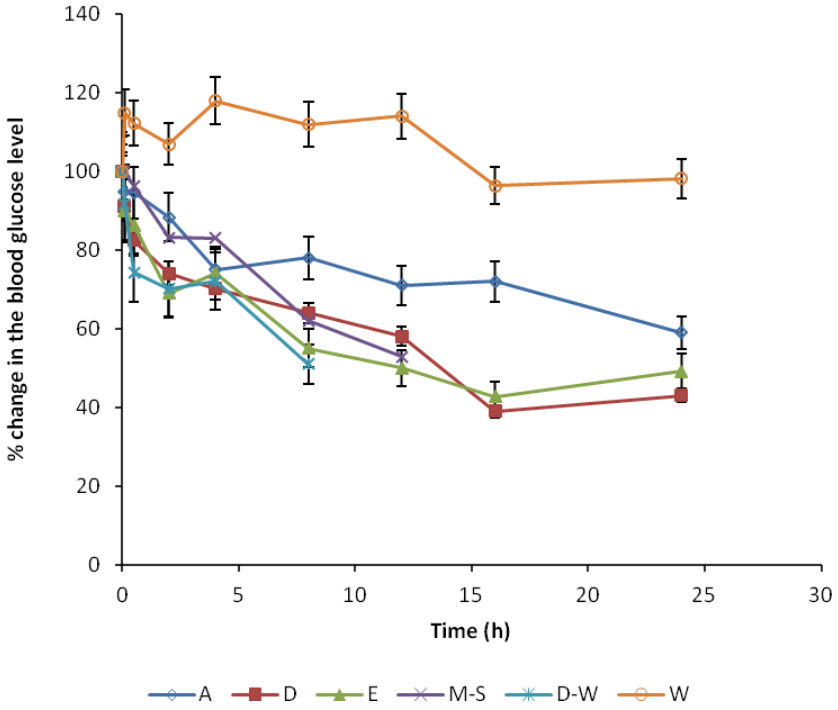

Figure 4. Effect of metformin loaded PEGylated-mucin on hyperglycaemic rats [A= (1:1) of PEG-Mucin, D = (3:1) of PEG-Mucin and E = (1:3) of PEG-Mucin, M-S = Marketed sample, D-W = Metformin dispersed in distilled water and W = distilled water]. Each of the batches (A, D and E) contained 500 mg of metformin HCl.

sample (M-S) and the dispersed pure metformin powder (D-W). All the metformin-loaded PEGylated-mucin matrix (A, D and E) prepared with the various ratio of mucin and PEG generally reduced blood glucose lowering effect higher than those of either marketed sample or and the dispersed metformin powder in distilled water. The high blood glucose reduction resulting from the formulations as compared to the positive controls indicates that there was effective delivery of the drug to the site where absorption takes place in the GIT. The formulation (batch E) produced high blood glucose reduction between 14 to 16 h after oral administration that was more than the marketed drug, and the effect lasted up to 24 h, while that of positive controls only lasted for 8 h. Batch D which contain PEG and mucin in the ratio of 3:1 show a similar effect to batch E, but with a slight reduction in the amount of glycemic effect (Figure 4). Batch A of the formulation PEG and mucin (1:1) significantly lowered the blood glucose level at the initial stage and declined within a short period of time.

Mucin being a viscoelasticity material would create a better adherence to the small intestine would lead to increased residence time, release at the delivery site, and would presumably affect the bioavailability of the drug. The mean turnover time of the mucous gel layer has been shown to vary between 47 and 270 min, which would indicate a significant factor in designing mucoadhesive drug-delivery systems (Des Rieux et al., 2006). The mucous gel layer itself represents an unstirred water layer which impedes drug, diffusion and adsorption across the epithelium. Consequently, a prolonged release effect as observed in all the formulations, but more in batch E with high concentration of mucin. However, the hydrophilicity nature of PEG may have contributed to the observed effect on the basal blood glucose reduction as seen in formulation D that contained high concentration of PEG ratio to mucin (3:1) in the *in vivo* study.

Recent studies have shown that factor such as hydrophilicity and mucoadhesion are capable of influencing drug release from the formulations and increase the transport of this system through the mucosa surfaces (Kenneth et al., 2010). In addition, the PEG chains are able to diffuse through the mucus and enhance mucoadhesion thereby enhance the release of the drug at the absorption site easier and faster than mucin (Kenneth et al., 2010).

Conclusion

PEGylated-mucin used in the study showed good

characterization based on the parameters measured. The *in vitro* model of the present study has proved to be reliable to predict release of metformin HCL through the uses of PEGylated-mucin matrix which was confirmed in the *in vivo* glucose lowering study. This method of drug delivery may be a promising technique in the area of drug discovery and development. PEGylated-mucin loaded metformin showed slightly higher release and maintained longer times when compared with immediate-release marketed products.

The glucose lowering effect was more in the formulations (C and D) than the commercially marketed metformin. This formulation could potentially reduce dosing frequency to once daily compared with two or three times daily for immediate-release formulations.

ACKNOWLEDGEMENTS

This work is a part of the thesis submitted for the award of Ph.D. by the Department of Pharmaceutics, University of Nigeria, Nsukka, Nigeria. The authors thank Farmex Meyer Pharma Ltd, Ikeja, Lagos State for the gift of metformin hydrochloride. Dr. Philip Builders of NIPRD is highly appreciated for his contribution. Dr. M. A. Momoh is grateful to the Islamic Education Trust Fund (IETF) and Nigeria-Saotome Principe for their financial support.

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African Journal of Pharmacy and Pharmacology Vol. 7(7), pp. 356-359, 22 February, 2013
Available online at http://www.academicjournals.org/AJPP
DOI: 10.5897/AJPP12.587
ISSN 1996-0816 © 2013 Academic Journals

Full Length Research Paper

Association of the p53 codon 72 polymorphism with breast cancer in central part of Iran

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Accepted 10 August, 2012

The tumor suppressor gene protein 53 (p53) plays a general role in cell cycle control, the initiation of apoptosis and in DNA repair. The human p53 gene is mutated and accumulated in more than 50% of cancers. Codon 72 exon 4 polymorphism (Arg72Pro) of the p53 gene has been implicated in cancer risk. This study was aimed at investigating the possible association between p53 Arg72Pro polymorphism and susceptibility to breast cancer among Iranian population. The p53 Arg72Pro genotypes were determined by polymerase chain reaction - restriction fragment length polymorphism (PCR-RFLP) analysis in 135 breast cancer cases and 150 controls. The PCR products were digested with *BstU I* restriction enzyme and the DNA fragments were then resolved by electrophoresis in 2% agarose gel. Out of the 135 breast cancer samples, 102 (75.55 %) samples were heterozygous (Arg/Pro), 27 (20%) samples homozygous for arginine (Arg/Arg) and 6 (4.45%) samples homozygous for proline (Pro/Pro). The frequencies of the three p53 genotypes; Arg/Pro, Arg/Arg and Pro/Pro in controls were 62, 24 and 14%, respectively. Heterozygosity for Arg/Pro of p53 codon 72 is potentially one of the genetic risk factors for breast cancer. The p53 Arg72Pro polymorphism may be used as a stratification marker in screening individuals at a high risk of breast cancer.

Key words: Breast cancer, protein 53 (p53), polymorphism, codon 72.

INTRODUCTION

Breast cancer is the most frequently encountered cancer in women throughout the world. Epidemiologic studies have suggested a number of risk factors, including genetic and environmental ones. The protein 53 (p53) tumor suppressor gene is the most involved genetic factor for breast cancer (Mabrouk et al., 2003). The gene for the tumor suppressor protein p53 occupies a central role in mediating cellular responses to DNA damage. Its activation results in either growth arrest in the G1 phase of the cell cycle or in apoptosis. The p53 gene contains a variety of polymorphisms and mutations (Zur Hausen, 2002; Aoki et al., 2009). Mutations in the p53 gene are associated with more than 50% of human cancers, and 90% of them affect p53-DNA interactions, and result in a partial or complete loss of transactivation functions (Zubo et al., 2009).

The p53 tumor suppressor gene, located on chromosome 17p13, is one of the most commonly mutated genes in all types of human cancer (He et al., 2011; Chen et al., 2008). The p53 tumor suppressor gene is frequently mutated in many forms of human carcinomas. A common polymorphism occurs at codon 72 of exon 4, with two alleles encoding either arginine (CGC) or proline (CCC). This p53 polymorphism is reportedly associated with cancer susceptibility. The distribution of the three genotypes (Arg/Arg, Arg/Pro and Pro/Pro) depends largely on the ethnic composition of the studied population (Omori et al., 2004). Recent studies showed the relation between p53 gene polymorphism with cancer of the stomach (Takeda et al., 2000), lung (Özbey et al., 2011), breast (Pich et al., 2000), ovary

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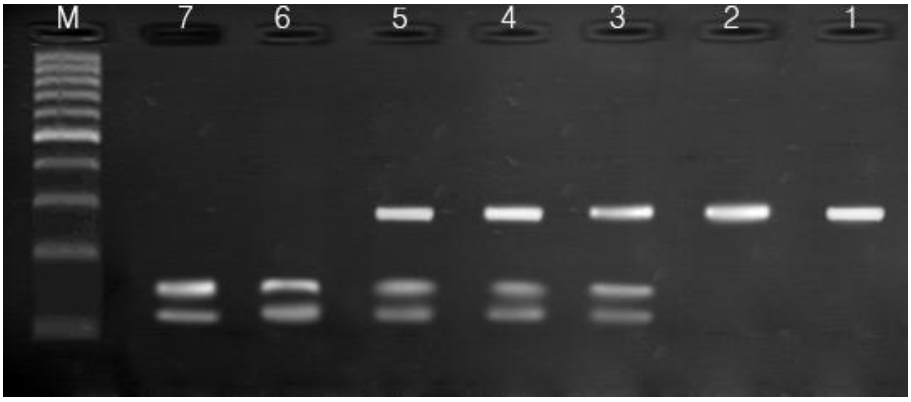

Figure 1. Genotype analysis by digestion of the amplified product and RFLP; lanes 1 and 2 are homozygote samples for Arg allele, lanes 3-4 are Arg/Pro heterozygote and lanes 6 and 7 are Pro homozygote samples. Lane M, 100 bp DNA ladder (Fermentas).

(Agorastos et al., 2004) and cervix (Jiang et al., 2010) among others in different parts of the world. In the present study, we examined the relation between the codon 72 polymorphism in p53 and the risk for breast tumorigenesis in Iranian patients.

MATERIALS AND METHODS

Sample collection and DNA extraction

Genomic DNA was purified from blood samples of 135 cases of breast cancer and 150 controls by DNA extraction kit according to the manufacturer's instructions. Samples were recruited from different hospitals of Isfahan (central part of Iran) during the period from February 2010 - October 2011. The isolated DNA was quantified by the agarose gel.

Primer, polymerase chain reaction (PCR) assay and restriction fragment length polymorphism (RFLP) analysis

PCR amplification was performed with two sets of primers: the outer oligonucleotide primers (forward: 5'-GCT CTT TTC ACC CAT CTA CAG -3'; reverse: 5'-TGA AGT CTC ATG GAA GCC AGC-3') and the inner oligonucleotide primers (forward: 5'-TCC CCC TTG CCG TCC CAA- 3'; reverse: 5'-CGT GCA AGT CAC AGA CTT-3') (Doosti et al., 2011).

The target sequence was amplified in a 50 µL reaction volume containing of genomic DNA, 0.2 mM dNTPs, 1X Taq buffer, 2 mM $MgCl_2$, 100 ng of each primer, and 1 unit of Taq DNA polymerase (Fermentas, Germany). The first round of PCR was carried out by applying a step-up program as follows: initial denaturation for 5 min at 94°C, followed by 30 cycles of 94°C for 1 min, 58°C for 1 min, and 72°C for 1 min, with a final extension for 5 min at 72°C. Two to 5 µL from the first round amplicon was used as a template for the second round PCR with the identical PCR program by inner oligonucleotide primers.

The *BstU I* restriction enzyme cuts within the sequence corresponding to the arginine codon (CGC) at position 72 to generate two visible fragments of 160 and 119 bp, leaving the proline allele uncut. A 20-µL of PCR product was digested overnight at 37°C in a 25 µL reaction volume containing 5 units of *BstU I* (Fermentas, Germany). After overnight digestion, the fragments were separated by electrophoresis on a 2% agarose gel and stained with ethidium bromide. Logistic regression analyses were used to adjust for possible confounding variables, and data were evaluated using the Pearson χ^2 tests.

RESULTS

Genotyping of the p53 codon 72 polymorphism was successful in 135 of 142 (95.07%) breast cancer cases and 150 of 158 (94.93 %) controls. Codon 72 arginine and proline alleles were investigated by PCR-based digestion analysis. The recognition site (CGCG) of the restriction enzyme was present only in the arginine-encoding allele. Thus, the proline allele is identified by the presence of a single fragment of 279 bp, and the arginine allele by two fragments of 160 and 119 bp, respectively. Heterozygous samples displayed all three fragments (Figure 1).

The frequency of the two alleles in breast cancer patients was compared with the frequency in the healthy control subjects. A significant difference in the genotype distribution was observed between the controls and patients with breast cancer. The distribution of the Arg/Arg, Arg/Pro and Pro/Pro genotypes in the control group was 36, 93 and 21 respectively. Allele frequencies in the control group were 0.55 for the Arg allele and 0.45 for the Pro allele. Among the patients, the frequencies of the Arg and Pro allele were 0.57 and 0.43 respectively. The corresponding distribution of the Arg/Arg, Arg/Pro and Pro/Pro genotypes in the patients was 27, 102 and 6, respectively (Table 1).

Our results indicate that individual heterozygous for the Arg allele has a higher risk of developing breast cancer than homozygotes and proline- homozygotes. A comparison of the genotype frequencies between breast cancer patients and normal controls confirmed the accumulation of Arg/Pro genotype in these patients ($P<0.01$).

Table 1. Genotype and allele frequencies of p53 in patients with breast cancer and controls.

Codon 72 genotypes	Breast cancer (n = 95.07%)	Controls (n = 94.93%)	*P*-value
Arg/Arg	27 (20)	36 (24)	<0.01
Arg/Pro	102 (75.55)	93 (62)	<0.02
Pro/Pro	6 (4.45)	21 (14)	>0.005
Arg Alleles	57	55	>0.10
Pro Alleles	43	45	<0.05

DISCUSSION

Although several studies have attempted to establish an eventual association between the polymorphism at codon 72 and human carcinogenesis, the issue is still a matter of controversy. Interestingly, a polymorphism has been demonstrated at codon 72, where the proline is frequently replaced by an arginine. Both forms are morphologically wild-type and do not differ in their ability to bind to DNA in a sequence-specific manner. However, there are a number of differences between these p53 variants in their abilities to bind components of the transcriptional machinery, to activate transcription, to induce apoptosis, and to repress the transformation of primary cells (Thomas et al., 1999). It has been shown that the codon 72 polymorphism (Arg-Pro polymorphism) of the common tumor suppressor p53 gene contributes to susceptibility to breast cancer. The Arg/Pro heterozygous genotype occurred more frequently in this cancer. The prevalence of the Arg/Pro genotype in breast cancer was higher than that of other genotypes.

In this study, we examined the prevalence of p53 codon 72 polymorphisms in a number of Iranian groups of breast cancer patients and controls. The prevalence of the Arg/Pro genotype in carcinoma cases was statistically different from that of the controls (75.55% versus 62%). Several studies have examined the role of the codon 72 polymorphism in mutation of the p53 gene in cancer. Langerod et al. (2002) identified p53 mutations more commonly in breast cancer from Arg/Arg homozygotes (28.5%), than among Arg/Pro heterozygotes (21%) or Pro/Pro homozygotes (4%). However, no difference in p53 mutation frequency among codon 72 genotypes has been reported in colorectal or bladder cancer (Furihata et al., 2002). Several studies have also suggested that the codon 72 Arg allele is preferentially mutated and retained in Arg/Pro heterozygotes (Tada et al., 2001). These authors have suggested that the codon 72 Arg containing mutants may have a selective growth advantage influencing the ratio of Arg and Pro containing mutants in tumors. Another study also observed the prevalence of homozygosity for the p53 Arg allele and a strong association between the Arg/Arg genotype in breast cancer patients (Buyru et al., 2003). Recently, higher number of arg allele was observed in breast cancer patients when compared to the controls suggesting that the arg allele may be associated with predisposing India women to breast cancer (Suresh et al., 2011).

CONCLUSIONS

In this study, the heterozygosity for Arg/Pro of p53 codon 72 was associated with breast cancer. Heterozygosity for Arg/Pro of p53 Arg72Pro is potentially one of the genetic risk factors for breast cancer in Iranian population. Therefore, the p53 Arg72Pro polymorphism may be used as a stratification marker in screening individuals at a high risk of breast cancer. In summary, the findings of the present study indicate that the p53 codon 72 polymorphism may be a genetic predisposing factor for breast adenocarcinomas, and p53 Arg 72 protein may be correlated with possible increased risk of this kind of cancers in central part of Iran.

ACKNOWLEDGEMENT

The authors are grateful to the competitors of Biotechnology Research Center of Islamic Azad University, Shahrekord Branch, for valuable discussion and useful suggestions of this work.

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African Journal of Pharmacy and Pharmacology Vol. 7(7), pp. 360-367, 22 February, 2013
Available online at http://www.academicjournals.org/AJPP
DOI: 10.5897/AJPP12.600
ISSN 1996-0816 ©2013 Academic Journals

Full Length Research Paper

Chemical composition, antioxidant activity and cytotoxicity of the essential oils of the leaves and stem of *Tarchonanthus camphoratus*

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Accepted 4 January, 2013

The aim of this study was to investigate the chemical composition, antioxidant potential and cytotoxicity of the essential oil of the fresh leaf, dry leaf and dry stem of *Tarchonanathus campharatus.* The antioxidant activity of the oils were examined by the 1,1-Diphenyl-2-picryl-hydrazil (DPPH), 2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) ($ABTS^{\cdot+}$), nitric oxide radical scavenging and reducing power methods. The brine shrimp lethality test and the MTT cytotoxicity test were used to investigate the cytotoxicity of the oils. Sesquiterpene hydrocarbons are the major components in the essential oil of the fresh leaves (36.46%) and of the dry leaves (59.18) whereas an aldehyde, butanal (35.77%) is the major component in the essential oil of the dry stem. The oils did not show significant antioxidant activity as evidenced by their high LC_{50} values in all the antioxidant assays. The cytotoxicity results indicated that the oils had low toxicity with LC_{50} values ranging from 400 to 900 µg/ml and 400 to 1100 µg/ml for the brine shrimp lethality test and MTT cytotoxicity assay respectively.

Key words: *Tarchonanthus camphoratus*, essential oil, antioxidant activity, cytotoxicity.

INTRODUCTION

In living systems, free radicals are constantly generated and when in excess they can cause extensive damage to tissues and biomolecules leading to various disease conditions, especially degenerative diseases, and extensive lysis (Halliwell and Gutteridge, 1998). The interest in antioxidants has been increasing because of their high capacity in scavenging free radicals which are related to various diseases (Silva et al., 2007). The most commonly used synthetic antioxidants; butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), propylgallate (PG) and test butylatedhydroquinone have been reported to cause liver damage and carcinogenesis (Sherwin et al., 1990). There is growing interest in natural antioxidants present in medicinal plants that might help attenuate oxidative damage (Silva et al., 2005; Muhammad et al., 2012). The health promoting effects of plants were found to be due to bioactive substances such as essential oils, flavonoids and phenolic compounds which have antioxidant activity (Liu, 2003; Komal et al., 2012).

Tarchonanathus campharatus L., (family Asteraceae) is a shrub of rarely more than six meters in height with a greyish appearance and occurs in a wide range of habitats (van Wyk et al., 1997). The strongly scented tree of *T. campharatus* has many medicinal applications in

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traditional healing mainly by smoking from burning leaves or by drinking infusions or decoctions. Traditionally, infusions and tinctures of the leaves are used for stomach trouble, headache, toothache, asthma, bronchitis, inflammation, rheumatism, venereal diseases, indigestion, heartburn, coughs, paralysis and cerebral haemorrhage (Hutchings et al., 1994; Anthony, 1999). The plant also shows powerful insect repellent action (Omolo et al., 2004; Essential oil newsletter, 2005).

In this study, the chemical composition, antioxidant potential and the cytotoxicity of the essential oils of the leaves and stem of *T. camphoratus* were investigated in order to find out their suitability as raw materials in food, pharmaceutical and industrial products.

MATERIALS AND METHODS

Plant material

Fresh materials of *T. camphoratus* were collected from Sangoyana in the northern part of Kwa-Zulu Natal province, South Africa during the month of March, 2010. The plant was identified by the local people during the time of collection and further identified by Mrs N.R Ntuli in the Department of Botany, University of Zululand. A Voucher specimen, (NSKN 1), was deposited at the University of Zululand herbarium. The fresh plant material was separated into leaves and the other part with leaves still attached to the stem was dried at room temperature.

Extraction of the essential oil

The fresh leaves, dry leaves and the dry stem were subjected to hydro-distillation using a Clevenger-type apparatus. The essential oils were collected 4 h after boiling, weighed and kept at 4^{o}C in sealed glass vials before analysis and bioassay.

Gas chromatography-mass spectrometry analysis

The GC-MS analysis was carried out using an Agilent 6890 GC with an Agilent 5973 mass selective detector [MSD, operated in the EI mode (electron energy = 70 eV), scan range = 45 to 400 amu, and scan rate = 3.99 scans/sec], and an Agilent ChemStation data system. The GC was equipped with a fused silica capillary HP-5 MS column of an internal diameter of 0.25 mm, film thickness 0.25 µm and a length of 30 m. The initial temperature of the column was 70°C and was heated to 240°C at a rate of 5°C/min. Helium was used as the carrier gas at a flow rate of 1 ml/min. The split ratio was 1:25. Scan time was 50 min with a scanning range of 35 to 450 amu. A 1%, w/v, solution of the samples in hexane was prepared and 1 µL was injected using a splitless injection technique.

Identification of components

The identification of the oil constituents was based on their retention indices determined by reference to a homologous series of *n*-alkanes (C_8-C_{30}), and by comparison of their mass spectral fragmentation patterns with those reported in the literature (Joulain and Koenig, 1998; Adams, 2007) and stored in the MS library [NIST database (G1036A, revision D.01.00)/ChemStation data system (G1701CA version C.00.01.080)]. The percentages of each component are reported as raw percentages based on the total ion current without standardization.

DPPH radical scavenging assay

50 µL of various dilutions of the essential oil (50 to 250, µg/ml) were mixed with 5 mL of a 0.004% methanol solution of DPPH and incubated for 30 min. The absorbance was measured against a corresponding blank at 517 nm. BHT (Sigma) was used as a positive control. Inhibition percentage of free radical DPPH was calculated in the following way:

% DPPH radical scavenging = [(control absorbance – sample absorbance)/control absorbance)] × 100

ABTS [2, 2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)] radical cation scavenging assay

The ABTS radical cation was generated by mixing 7 mM of ABTS solution with 2.45 mM. Potassium persulfate was left to stand in the dark for 16 h at room temperature. 1 ml of ABTS was added to 1 ml of different essential oil concentrations (25 to 250 µg/ml) and absorbance of the mixture was measured at 734 nm after 6 min.

% inhibition = [(Control absorbance – Sample absorbance)/Control absorbance)] × 100

Nitric oxide radical inhibition assay

The reaction mixture containing 2 ml sodium nitroprusside (10 mM), 0.5 ml phosphate buffer saline (pH 7.4) and 0.5 ml of different concentrations of the essential oil (50 to 250, µg/ml) or standard solution (ascorbic acid, 0.5 ml) was incubated at 25ºC for 150 min. Then 1 ml of sulfanilic acid reagent (0.33% sulfanilic acid in 20% glacial acetic acid) was added to 0.5 ml of reaction mixture for 5 min to complete diazotization and 1 ml naphthyl ethylene diamine dihydrochloride was added and allowed to stand for 30 min at 25ºC. The absorbances of these solutions were measured at 540 nm (Badami et al., 2005).

% Nitric oxide scavenged = [(Control absorbance – Sample absorbance)/Control absorbance)] × 100

Total reducing power

The reducing power was determined according to the method of Oyaizu (1986). Different concentrations, (25 to 250 µg/ml), of the essential oils in methanol (2.5 ml) were mixed with 2.5 ml of 0.2 M sodium phosphate buffer (pH 6.6) and 2.5 ml of 1% potassium ferricyanide. The mixture was incubated at 50°C for 20 min followed by addition of 2.5 ml of 10% trichloroacetic acid (TCA) and centrifuged at 1000 rpm for 10 min. 2.5 ml of the mixture was mixed with 2.5 ml of deionised water and 0.5 ml of 0.1% ferric chloride and its absorbance measured at 700 nm against a blank. Ascorbic acid was used as the reference standard. To determine reducing power, 2.5 ml of the mixture was mixed with 2.5 ml of deionised water and 0.5 ml of 0.1% ferric chloride and its absorbance measured at 700 nm. Ascorbic acid was used as the reference standard. Higher absorbance of the reaction mixture indicates greater reducing power.

Brine shrimp cytotoxicity assay

The brine shrimps were hatched in sea water for 48 h at room

temperature. The nauplii (harvested shrimps) were attracted to one side of the vessel with a light source. The essential oil were prepared at 1000, 500, 100 and 10 µg/ml (each test in triplicates) in 0.02% Tween 80. The essential oil (0.5 ml) was introduced in a test-tube and sea water (4 ml) added. Ten shrimps per test tube were added for each concentration and made up to 5 ml with sea water.

Potassium dichromate was used as positive control. The negative control was 0.02% Tween 80 (5 ml). The surviving larvae were counted after 24 h and the percent deaths at each dose and positive control were determined.

Cytotoxicity analysis by the MTT assay

The MTT assay was done using two cell lines, human embryonic kidney cells and human hepatocellular carcinoma cells. The cells were grown to confluenecy in 25 cm^3 flasks. This was then trypsinised and plated into 48 well plates at specific seeding densities. Cells were incubated overnight at 37°C. The medium was removed and fresh medium (MEM + Glutmax + antibiotics) was added. Extracts (50 to 100 µg) were added in triplicate and incubated for 4 h. The medium was again removed and replaced by a complete medium (MEM + Glutmax + antibiotics + 10% Fetal bovine serum). After 48 h the cells were subjected to MTT [3-(4,5-dimethylthiazolyl)-2,5-diphenyl-tetrazolium bromide] assay. Briefly, the medium was removed from the cells and 200 µl of 5 mg/ml MTT in phosphate buffered saline (PBS) as well as 200 µl of medium were added to each well containing cells. The multiwell plate was incubated for 4 h and thereafter the medium and MTT were removed and 200 µl of DMSO was added to each well and incubated at 37°C for 10 min. Absorbance of the dissolved solutions were read using a Mindray Plate Reader at 570 nm. The cytotoxicity was calculated after comparing with the control. The control consisted of cells without the extract.

Statistical analysis

Results of antioxidant activity are presented as means ± SD of three measurements. Data were evaluated through regression analysis using QED statistics program and IC_{50} values, where applicable, were determined by linear regression. Means between treatments were compared by Tukey's Studentized Range Test using one way ANOVA.

RESULTS AND DISCUSSION

Chemical composition

Yellowish green oils with yields of 0.14, 0.09 and 0.03% were obtained from the fresh leaves, dry leaves and dry stem of the plant respectively. The oils gave a total of 33, 27 and 25 identified compounds representing 85.9, 73.01 and 72.66% of the total oil composition from the fresh leaves, dry leaves and dry stem respectively (Table 1). The major compounds in the oil of the fresh leaves were; α-cadinol (9.40%), 1,8 cineole (9.19%), δ-cadinene (6.89%), butanal (6.10%) and caryophyllene oxide (4.21%) while in the dry leaf oil were; β-guaiene (10.7%), γ-cadinene (9.09%), δ-cadinene (6.80%), aromandrene (6.12%), β-caryophyllene (5.48%) and γ-muurolene (5.13%). In the dry stem oil, butanal (35.77%), T-muurolol (10.33%) and δ-cadinene (5.54%) featured as the major compounds. Sesquiterpene hydrocarbons dominated the oils of the fresh and dry leaves.

DPPH radical scavenging assay

The radical scavenging activity of the essential oils of *T. camphoratus* was determined from the reduction in absorbance at 517 nm due to scavenging of the stable DPPH radical. DPPH is a stable nitrogen-centred free radical the colour of which changes from violet to yellow upon reduction by either the process of hydrogen or electron donation. The oils showed a relatively weak dose dependent inhibition of DPPH activity, with high LC_{50} of 12578.89, 9942.08 and 7010.03 µg/mL for fresh leaves, dry leaves and dry stem respectively (Table 2). The LC_{50} values of the oils were not comparable to that of the standard BHT at $p \leq 0.05$.

$ABTS^{\bullet+}$ radical scavenging assay

The ABTS radical cation is reactive towards most antioxidants and the decolorization of the $ABTS^{\bullet+}$ radical reflects the capacity of an antioxidant species to donate electrons or hydrogen atoms to inactivate this radical species (Re et al., 1999). The results for percent scavenging at different concentrations and LC_{50} values for the oils are shown in Table 3. There was a significant difference in the means of the oils and that of BHT at $p \leq 0.05$. The high LC_{50} values of the oils suggest poor $ABTS^{\bullet+}$ radical scavenging activity.

Nitric oxide (NO) assay

In this assay, the ability of the essential oils to counteract the oxidation of nitric oxide with oxygen and reduce the production of nitrite ions which act as free radicals was investigated.

Table 4 shows the %inhibition of nitric oxide generation by the essential oils and of the standard ascorbic acid. The activity of the standard ascorbic acid was more pronounced with LC_{50} value of 210.50 µg/ml when compared to LC_{50} values of the essential oils at $p \leq 0.05$.

Total reducing power

In the reducing power assay, the presence of antioxidants in the samples results in the reduction of Fe^{3+} to Fe^{2+} by donating an electron. The method evaluates the ability of plant extracts to reduce potassium ferricyanide solution which is monitored by measuring the formation of Perl's Prussian blue at 700 nm. Increasing absorbance at 700 nm indicates an increase in reductive ability. Figure 1 shows dose-response curves for the reducing powers of the essential oil. It was found that the reducing power

Table 1. Chemical constituents of the essential oils of the fresh leaves, dry leaves and dry stem of *T. camphoratus*.

Name of compound	Percentage composition			
	KI	FL	DL	DS
Monoterpene hydrocarbons		**9.57**	**1.61**	**0.00**
α- Pinene	938	2.52	0.45	
Camphene	952	2.15	0.33	
β- Pinene	978	0.65		
α-Terpinene	1017	2.61	0.65	
p-Cymene	1026	1.64	0.18	
Oxygenated monoterpenes		**11.79**	**6.26**	**0.79**
1,8-Cineole	1033	**9.19**	1.94	0.15
Linalool	1098	1.42	1.77	0.33
Camphor	1145		0.62	
(-)-Borneol	1169			0.07
Terpinene-4-ol	1180	0.56	0.43	0.08
(-)-α-Terpineol	1190	0.62	0.82	0.16
Carvacrol	1299		0.68	
Sesquiterpene hydrocarbons		**36.46**	**59.18**	**18.54**
α-Copaene	1378	1.45	2.33	1.21
Isocomene	1392			1.35
α-Elemene	1393		2.98	
Calarene	1403	2.02	3.60	
δ-gurjunene	1410	1.25		
(-)-Isoledene	1419	1.22	2.72	0.63
Beta-caryophyllene	1427	2.73	**5.48**	1.05
α-gurjunene	1436			0.13
α-Guaiene	1439		2.73	0.86
α-humulene	1461	0.64	0.97	0.29
γ-gurjunene	1472		0.43	
Aromandrene	1475	2.05	**6.12**	0.16
α-Muurolene	1476	3.50		1.35
γ-Muurolene	1480	3.19	**5.13**	2.11
Ledene	1482	0.93		
Germacrene D	1484			0.69
Eremophilene	1486	1.25	0.10	1.04
α-Selinene	1488	1.08		
Valencene	1491	2.39		
β-Guaiene	1500	1.20	**10.70**	
γ-Cadinene	1513	1.50	**9.09**	2.13
cis-calamenene	1520	3.17		
δ-Cadinene	1526	**6.89**	**6.80**	**5.54**
Oxygenated sesquiterpenes		**19.5**	**3.19**	**14.26**
Elemol	1549	**3.5**	2.76	
Spathulenol	1578		0.43	
Caryophyllene oxide	1580	**4.21**		2.90
Hinesol	1632	2.39		
t-Cadinol	1640			1.03
t-Muurolol	1642			**10.33**
α-cadinol	1650	**9.40**		

Table 1. Contd.

Others		**8.58**	**2.77**	**39.07**
Butanal	620	**6.10**	2.77	**35.77**
Isoaromadendrene epoxide	1579	1.5		
α-Costol	1801	0.98		
Hexadecanoic acid	2117			**3.30**

Table 2. DPPH radical scavenging assay of the essential oils from the fresh leaves, dry leaves and dry stem of *Tarchonanthus camphoratus.*

Concentration (µg/ml)	%scavenging activity			[d]BHT
	[a]Fresh leaf oil	[b]Dry leaf oil	[c]Dry stem oil	
25	0.00	0.00	0.00	57.47
50	0.00	3.13	4.87	82.03
100	7.73	9.00	7.50	92.87
150	11.13	13.47	10.85	94.80
200	15.90	16.67	14.20	96.80
250	18.47	19.75	26.55	99.10

a. Linear equation: y = 19.57X – 30.23 LC_{50} = 12578.89[a]
b. Linear equation: y = 19.82X – 29.23 LC_{50} = 9942.08[b]
c. Linear equation: y = 21.26X – 31.76 LC_{50} = 7010.03[c]
d. Linear equation: y = 38.77X + 9.81 LC_{50} = 10.88[d]

Table 3. $ABTS^{\bullet+}$ radical scavenging assay of the essential oils from the fresh leaves, dry leaves and dry stem of *Tarchonanthus camphoratus.*

Concentration (µg/ml)	%scavenging activity			[d]BHT
	[a]Fresh leaf oil	[b]Dry leaf oil	[c]Dry stem oil	
25	0.00	3.10	5.60	38.30
50	3.80	14.90	18.70	65.00
100	7.60	20.20	23.70	80.00
150	10.30	23.60	25.40	82.00
200	13.80	27.10	29.40	85.00
250	17.60	29.60	32.70	88.20

a. Linear equation: y = 18.81X – 29.16 LC_{50} = 16158.45[a]
b. Linear equation: y = 20.87X – 21.05 LC50 = 2539.42[b]
c. Linear equation: y = 19.01X – 14.23 LC50 = 2391.93[c]
d. Linear equation: y = 31.55X + 13.32 LC50 = 14.54[d]

of the essential oil of the dry stem, fresh and dry leaves of *T. camphoratus* was much lower than that of the standards, ascorbic acid and BHT.

Cytotoxicity assay

The LC_{50} values of the essential oil of the fresh leaves, dry leaves and dry stem were 889.0, 676.8, 442.9 µg/ml respectively and for the standard, potassium dichromate, 3.44 µg/ml (Table 5). There was a significant difference in the means of percent mortality of the essential oils and of the standard, potassium dichromate ($p \leq 0.05$). There was no significant difference in the activity of the essential oils against the brine shrimps ($p \leq 0.05$). The MTT assay is a well established method to assess mitochondrial competence (Freshney, 2000). Using this assay, we assessed the ability of the essential oils of the fresh leaf, dry leaf and dry stem to suppress mitochondrial respiration in human embryonic kidney cells and human hepatocellular carcinoma cells. The LC_{50} values of the essential oils used in this study in both cell lines were above 100 µg/ml (Table 6). The results revealed that there was no significant difference, ($p \geq 0.05$), in the

Table 4. Nitric oxide scavenging activity of the essential oils from the fresh leaves, dry leaves and dry stem of *Tarchonanthus camphoratus.*

Concentration (µg/ml)	% scavenging activity			[d]BHT
	[a]Fresh leaf oil	[b]Dry leaf oil	[c]Dry stem oil	
125	6.88	5.76	11.58	41.56
250	11.48	5.94	24.15	50.49
500	13.07	6.71	26.68	68.40
750	19.17	6.88	27.14	73.80
1000	24.74	27.51	28.95	89.30
1250	32.46	39.42	36.52	89.80

a. Linear equation: y = 22.71X – 43.22 LC_{50} = 12729.16[a]
b. Linear equation: y = 19.79X – 27.49 LC_{50} = 8735.82[b]
c. Linear equation: y = 27.77X – 59.45 LC_{50} = 8234.05[c]
d. Linear equation: y = 50.92X – 68.30 LC_{50} = 210.50[d]

Table 5. Inhibitory effects of the essential oils from the fresh leaves, dry leaves and dry stem of *Tarchonanthus camphoratus* on brine shrimps.

Concentration (µg/ml)	% inhibition			[d]Potassium dichromate
	[a]Fresh leaf oil	[b]Dry leaf oil	[c]Dry stem oil	
10	0.00	0.00	0.00	93.33
100	6.67	10.00	13.33	100.00
500	33.33	36.67	43.33	100.00
1000	66.67	70.00	76.67	100.00

a. Linear equation: y = 30.14x – 38.88 LC50 = 889.0[a]
b. Linear equation: y = 31.79x – 39.98 LC50 = 676.78[b]
c. Linear equation: y = 35.35x – 43.55 LC50 = 442.99[c]
d. Linear equation: y = 3.31x + 91.13 LC50 = 3.44[d]

Table 6. Cytotoxicity of the essential oils from the fresh leaves, dry leaves and dry stem of *Tarchonanthus camphoratus* on human embryonic kidney cells and hepatocellular carcinoma cells.

Plant parts	LC_{50}	Concentration (µg/ml)
	Hepatocellular carcinoma cells	Embryonic kidney cells
Fresh leaves	774.80	1042.15
Dry leaves	438.39	708.28
Dry stem	356.07	438.44

The LC_{50} values were expressed as the mean ±S.D determined from the results of MTT assay in triplicate experiments.

action of the essential oils of the dry stem, fresh and dry leaves on each of the human cells used.

DISCUSSION

Antioxidant

In all the antioxidant assays carried out in this study, the essential oils of the dry leaves, fresh leaves and dry stem of *T. camphoratus* showed poor antioxidant activity. A good correlation between the phenolic content in plants and their antioxidant activity has been reported (Tawaha et al., 2007; Othman et al., 2007; Nadeem et al., 2012). Essential oils rich in monoterpene hydrocarbons have also been reported to have high antioxidant activity (Tepe et al., 2005). The poor antioxidant activity of these essential oils, probably, is due to their lack of phenolic contents and low concentrations of monoterpene hydrocarbons. However, the low values of antioxidant and reducing power may not imply low medicinal value.

Emerging trends in antioxidant research point to the fact

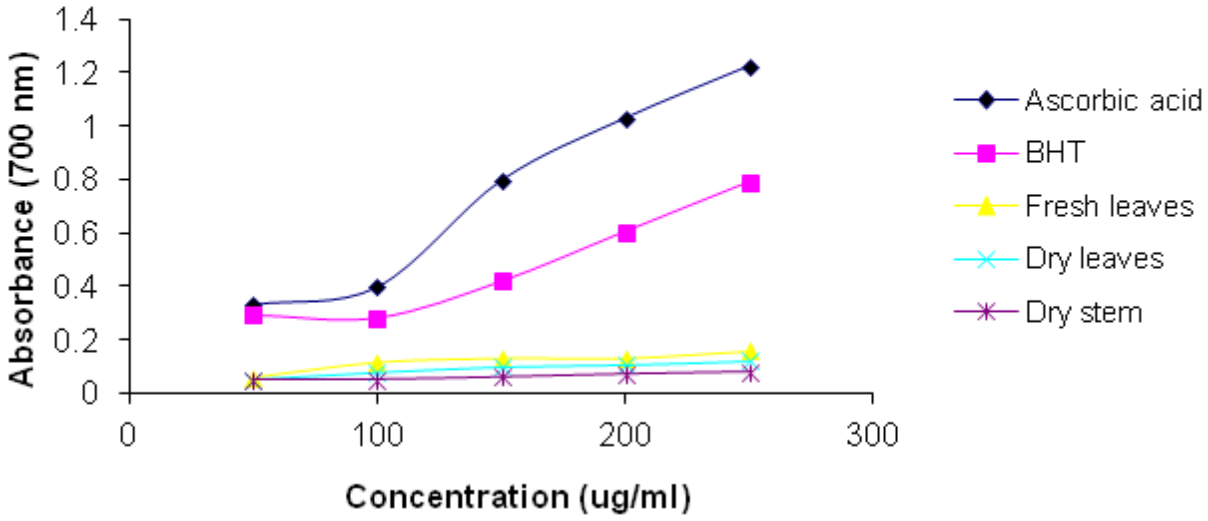

Figure 1. Total reducing power of the essential oils of the fresh leaves, dry leaves and dry stem of *Tarchonanthus camphoratus.* Ascorbic acid and BHT were used as the positive control.

that low levels of phenolics and other phytochemicals plus low value of antioxidant indices in plants do not translate to poor medical properties (Makari et al., 2008; Nasir et al., 2011).

Cytotoxicity

One indicator of a toxicity of a substance is LC_{50} which is the amount of a substance that kills 50% of the test organisms. All the essential oils investigated in this study, were found to have LC_{50} values > 30 µg/ml. According to the American National Cancer Institute, the LC_{50} limit to consider for a crude extract promising for further purification to isolate biologically active (toxic) compounds should be lower than 30 µg/ml (Suffness and Pezzuto, 1990). Other authors suggest that oils and extracts from plants presenting LC_{50} values below 1000 µg/ml are known to contain physiologically active principles (Meyer et al., 1982). The essential oils investigated showed low + antioxidant activities but do have some physiologically active principles.

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African Journal of Pharmacy and Pharmacology Vol. 7(7), pp. 368-374, 22 February, 2013
Available online at http://www.academicjournals.org/AJPP
DOI: 10.5897/AJPP12.1179
ISSN 1996-0816 ©2013 Academic Journals

Full Length Research Paper

Neuronal activities of berberine in *Schistosoma mansoni*-infected mice

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Accepted 7 January, 2013

The present study aimed to investigate the neuronal activities of berberine (BER) against *Schistosoma mansoni*-induced infection in mice. Animals were divided into four groups. Group I served as a vehicle control. Group III was gavaged with 100 µl of 12 mg/kg berberine chloride for 10 days. Group II and Group IV were infected with 100±10 *S. mansoni* cercariae, and on day 46 p.i. with *S. mansoni*. The animals of Group IV received 100 µl berberine chloride by gavage once daily for 10 days at a dose of 12 mg/kg body weight. All mice were sacrificed at day 55 post-infection. Schistosomiasis induced a highly significant reduction in contents of epinephrine (E), norepinephrine (NE), dopamine (DA), serotonin (5-HT), 5-hydroxyindole acetic acid (5-HIAA). On contrary, schistosomiasis resulted in a highly significant increment in the contents of calcium (Ca^{2+}), magnesium (Mg^{2+}), sodium (Na^{+}) and chloride (Cl^{-}) ions. Moreover, BER treatment induced a highly significant decrease in most investigated parameters. Collectively, BER could be considered as a neuro-modulator of *S. mansoni*-infected mice brain.

Key words: *Schistosoma mansoni*, Berberine, neurotransmitters, ions, mice.

INTRODUCTION

Schistosomiasis (bilharziasis) is a parasitic disease caused by blood flukes of the genus *Schistosoma.* It is the second most significant parasitic disease in the world after malaria (Fiore et al., 2002). More than 200 million people worldwide are infected, with 600 million people exposed to the risk of infection (Carod-Artal, 2008). *Schistosoma mansoni* is endemic in the Middle East (Egypt, Iraq), South America, the Caribbean, sub-Saharan Africa and southern areas of Africa. The infection is known to induce granulomas, not only in the liver and intestine, but also in the brain due to the presence of eggs, resulting in neuropathological and psychiatric disorders (Aloe and Fiore, 1998). Neuroschistosomiasis is referred to the schistosomal involvement of the central nervous system (Ferrari and Moreira, 2011). Fiore et al. (1998) reported that schistosome infection produced body weight reduction, increased analgesia, induced anxiety and decreased locomotion in mice.

For schistosomiasis, vaccine is nonexistent and drugs remain the mainstay of disease control. However, the current drug index is limited and/or inadequate and the problem is being further exacerbated by the emergence of drug resistance (Ismail et al., 2002; Silva et al., 2003). This raises a need for complementary and alternative drugs that are both effective and safe (Jatsa et al., 2009).

Attention has been focused on the protective effect of naturally occurring antioxidants, generally in biological systems (Asgarpanah and Ramezanloo, 2012; Nasri et al., 2012) and specifically against Shistosoma (Orledge et al., 2012). Berberine (BER) is a plant alkaloid with a long history of medicinal use in both Ayurvedic and Chinese medicine (Kulkarni and Dhir, 2008; Bhutada et al., 2010). Previous studies have shown that BER has a wide ranging pharmacological and biological activities including anthelmintic (Birdsall and Kelly, 1997), anti-

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inflammatory (Ivanovska and Philipov, 1996), anti-amnesic (Peng et al., 1997), anxiolytic (Peng et al., 2004), anti-depressant, analgesic and neuroprotective activities (Bhutada et al., 2010). All these effects of BER might be attributed to its capacity to modulate several neurotransmitters such as serotonin (Kong et al., 2001; Castillo et al., 2005). The present study aimed to investigate the neuronal activity of berberine (BER) against *S. mansoni*-induced infection in mice.

MATERIALS AND METHODS

Animals

Thirty two male Swiss albino mice were bred under specified pathogen-free conditions and fed a standard diet and water *ad libitum*. The experiments were performed only with male mice at an age of 9 to 11 weeks and were approved by state authorities and followed Egyptian rules for animal protection.

Infection of Mice

S. mansoni cercariae were from Schistosome Biological Supply Center at Theodor Bilharz Research Institute, Imbaba, Giza, Egypt. Mice were exposed to 100±10 *S. mansoni* cercariae per mouse by the tail immersion method, modified by Oliver and Stirewalt (1952).

Experimental design

Animals were allocated to four groups of eight mice each. Group I served as a vehicle control and received water (100 µl water/mouse) by oral administration for 10 days. Group III was gaveged with 100 µl of 12 mg/kg berberine chloride (Sigma, St. Louis, MO, USA) (Jahnke et al., 2006) for 10 days. Group II and Group IV were infected with 100±10 *S. mansoni* cercariae, and on day 46 p.i. with *S. mansoni.* The animals of Group IV received 100 µl berberine chloride by gavage once daily for 10 days at a dose of 12 mg/kg body weight. On day 55 p.i with *S. mansoni*, the animals of all groups were cervically dislocated. Brains were rapidly excised from skulls, blotted and chilled. The brain tissue was weighed, wrapped in plastic films and quickly stored at -70°C until used for estimation of the epinephrine (E), norepinephrine (NE), dopamine (DA), serotonin (5-HT), 5-hydroxyindole acetic acid (5-HIAA) according to the method of Ciarlone (1978). The levels of calcium (Ca^{2+}), magnesium (Mg^{2+}), sodium (Na^{+}) and chloiride ions (Cl-) were estimated according to Murphy (1987).

Statistical analysis

One way analysis of variance (ANOVA) according to Duncan's test (1955) was performed using the Statistical Package for the Social Science (SPSS) version 13. The Kolmorogov-Smirnov test was used to test for normal distribution of parameters. The histograms were drawn using Microsoft Excel. The percentage change was calculated as:

$$\% \text{ Change} = \frac{\textit{Mean of treated - Mean of control}}{\textit{Mean of control}} \times 100$$

RESULTS

Our data indicated that berberine treatment for 10 days has a pronounced effect of the level of the brain neurotransmitters. Infection of mice with *S. mansoni* induced a neuronal alterations in neurotransmitters as indicated with a significant decrease ($P \leq 0.05$) in the brain epinephrine, norepinephrine, dopamine, sertonine and 5-hydroxyindol acetic acid with a percentage change of approximately – 54% (Figure 1), -47% (Figure 2), -93% (Figure 3), -39% (Figure 4) and -65% (Figure 5), respectively.

BER was able to significantly ($P \leq 0.05$) lower the increased reduction in the level of dopamine (Figure 3), sertonine (Figure 4) and 5-hydroxyindol acetic acid (Figure 5) due to *S. mansoni* infection. However, the level of brain epinephrine (Figure 1) and norepinephrine (Figure 2) was not significantly changed after treatment of the infected mice with BER.

One aim of the present study was to identify infection associated changes of trace elements in the brain during the course of *S. mansoni* infection in mice. Table 1 showed the clear significant increase in the percentage of calcium in the brain of the infected mice compared to the vehicle control. BER treatment induced a highly significant decline in Ca^{2+} ion content versus the infected (-BER) group indicating the ameliorative role of BER on the increased level of Ca^{2+} ion content induced by *S. mansoni* infection. Also, compared to the vehicle control, the level of Mg^{+}, Na^{+}, and Cl^{-} was significantly increased due to infection of mice with *S. mansoni* (Table 1) with a percentage change of approximately 37, 12 and 275%, respectively. Again berberine could significantly ($P \leq 0.05$) improve the induced alterations in these minerals due to infection (Table 1).

DISCUSSION

Berberine induced a neuronal activity against schistosomiasis and could be considered as a neuro-modulator of *S. mansoni*-infected mice brain. Neuroschistosomiasis mansoni, referring to *S. mansoni* involvement of the central nervous system (CNS), may or may not result in clinical manifestations (Ferrari et al., 2008). Clinical studies have shown that humans affected with neuro-schistosomiasis suffer pain in the limbs and changes in peripheral sensory responses (Scrimgeour and Gajdusek, 1985).

Our results showed a reduction in E, NE, DA, 5-HT and 5-HIAA contents due to *S. mansoni* infection; on contrary, Ca^{2+}, Mg^{2+}, Na^{+} and Cl^{-} ions contents showed an increment in brain tissue. Several studies reported that schistosome infection induce epilepsy, seizures, cognitive impairments, deficits in learning abilities and behavioral changes (Katchanov and Nawa, 2010; Ferrari and

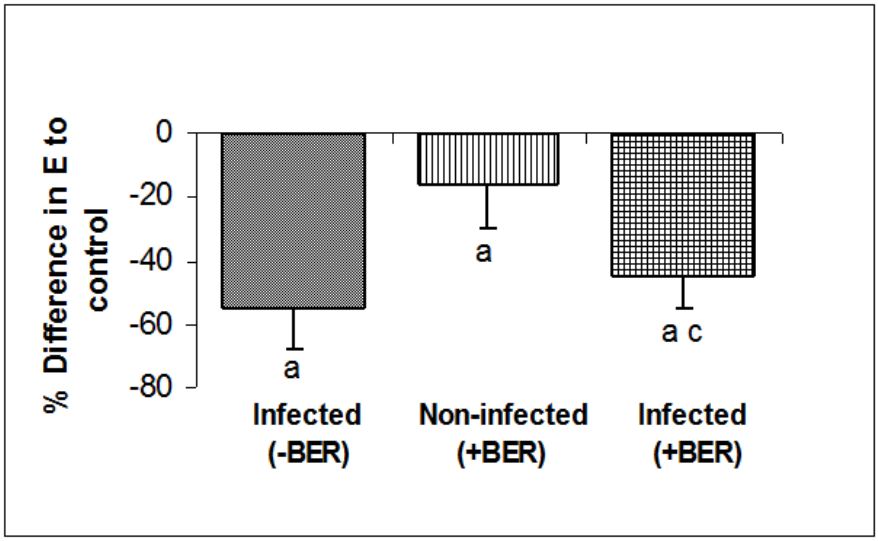

Figure 1. Berberine (BER) induced alterations in brain epinephrine (E) (µg/g tissue) of mice infected with *S. mansoni.* Values are means ± SE. a: Significant against non-infected (-BER) group at *P*≤0.05, b: Significant against infected (-BER) group at *P*≤0.05, c: Significant against non-infected (+BER) group at *P*≤0.05.

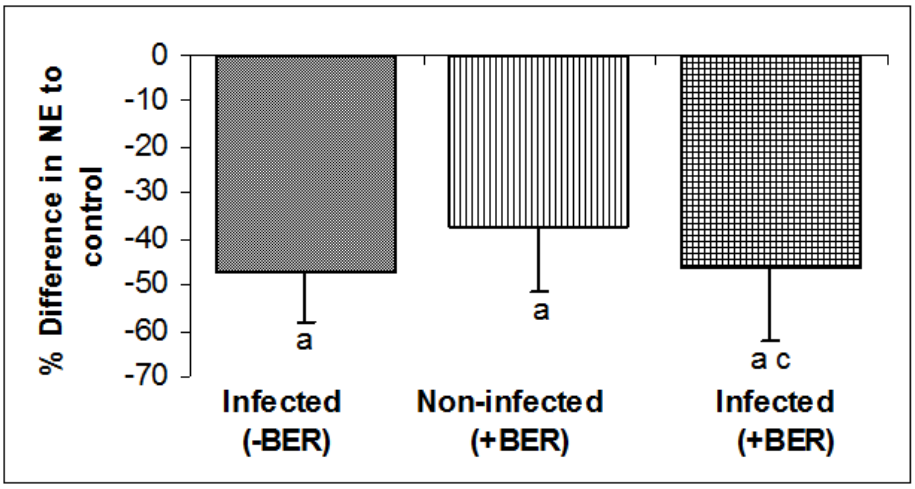

Figure 2. Percentage change in norepinephrine (NE) (µg/g tissue) in brain of mice infected with *S. mansoni* and treated with Berberine (BER). Values are means ± SE. a: Significant against non-infected (-BER) group at *P*≤0.05, b: Significant against infected (-BER) group at *P*≤0.05, c: Significant against non-infected (+BER) group at *P*≤0.05.

Moreira 2011). Some authors attributed the behavioral dysfunctions and the different types of seizures to deficiencies in the activities of the NE, DA and 5-HT systems (Applegate et al., 1986; Trindade-Filho et al., 2008).

The increment in Ca^{2+}, Mg^{2+} and Na^{+} ions concentration in brain tissue may be due to, in part, to an increase in the intracellular Na^{+} flow by binding to the Na^{+} ions channels and prolonging the opening time. Moreover, Mg^{2+} chelation is leading to prolong the opening time of the Ca^{2+} ion channel, thus increasing intracellular Ca^{2+} ions concentration (Davies and Maesen, 1989), this increase in intracellular Ca^{2+} ions concentration led to the rupture of the vesicles in the presynaptic terminals and increase the release of the neurotransmitters by exocytosis (Bullock et al., 1995) as a result the content of catecholamine is decreased in the present results. Moreover, the dysregulation in Cl^{-} homeostasis occurs in

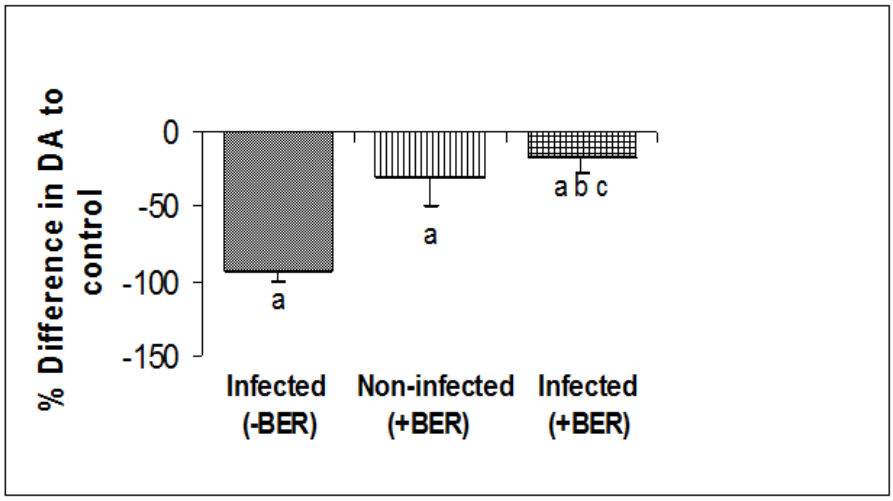

Figure 3. Changes in dopamine (DA) (µg/g tissue) percentage in brain of mice infected wit *S. mansoni* and treated with berberine (BER). Values are means ± SE. a: Significant against non-infected (-BER) group at *P*≤0.05, b: Significant against infected (-BER) group at *P*≤0.05, c: Significant against non-infected (+BER) group at *P*≤0.05.

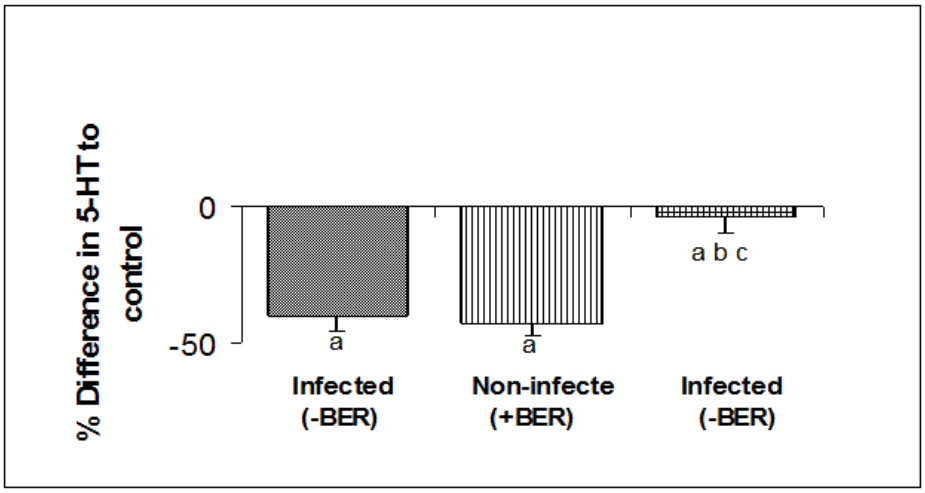

Figure 4. Changes in the serotonin (5-HT) (µg/g tissue) content in brain homogenate of mice infected with *S. mansoni* and treated with berberine (BER). Values are means ± SE. a: Significant against non-infected (-BER) group at *P*≤0.05, b: Significant against infected (-BER) group at *P*≤0.05, c: Significant against non-infected (+BER) group at *P*≤0.05.

many CNS pathologies including epilepsy and chronic pain (Asiedu et al., 2012). It was known that the enhancement of $GABA_A$ and $GABA_C$ receptors that are gated by Cl^- ion content, leading to increase the release of GABA and influx of Cl^- ion causing an increase in Cl^- ion content in brain tissue as a result Cl^- ion content was increased in brain tissue in this study. Thus, schistosome infection induces analgesia through the activation of Cl^- channel coupled GABA receptors.

Hu et al. (2012) reported that BER can cross the blood brain barrier, enter the cell and interact with DNA to act as a neuroprotectant. Several studies stated that BER produces anxiolytic (through modulation of 5-HTergic system), analgesic and antipsychotic (through modulation of DAergic system), antidepressant (through modulation of 5-HTergic, adrenergic and DAergic). Interestingly, modulators of these neurotransmitters are reported to implicate in anticonvulsant activity (Bhutada et al., 2010; Lee et al., 2010). BER gavage to schistosome infected mice group resulted in a highly significant reduction in E,

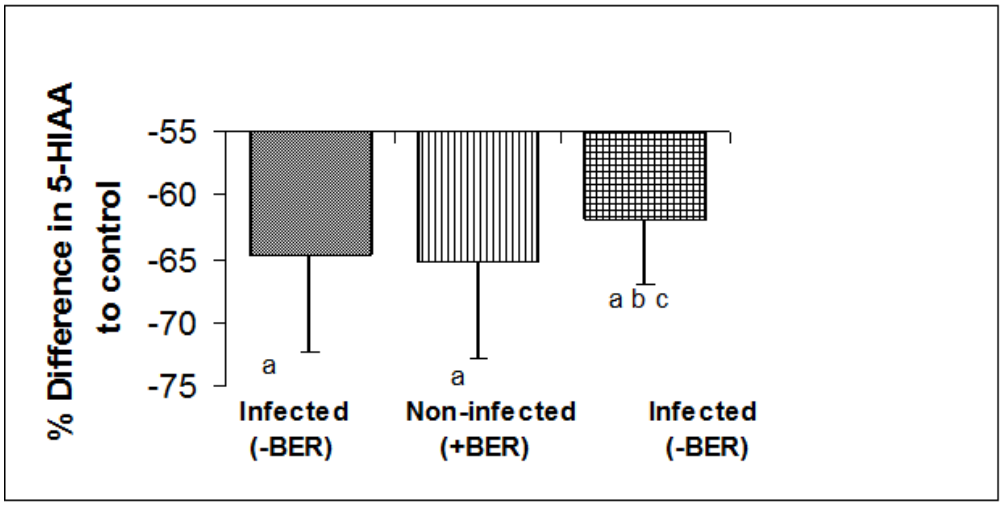

Figure 5. 5-hydroxyindole acetic acid (5-HIAA) (µg/g tissue) content in infected mice brain tissue with *S. mansoni* and treated with berberine (BER). Values are means ± SE. a: Significant against non-infected (-BER) group at $P \leq 0.05$, b: Significant against infected (-BER) group at $P \leq 0.05$, c: Significant against non-infected (+BER) group at $P \leq 0.05$.

Table 1. Changes in Ca^{2+}, Mg^{2+}, Na^{+} and Cl^{-} contents in brain of mice infected with *S. mansoni* and treated with Berberine (BER).

Group	Ca^{2+} (mg/g)	Mg^{2+} (mg/g)	Na^{+} (mg/g)	Cl^{-} (mg/g)
Non-infected (- BER)	33.63 ± 2.4	22.58 ± 1.0	141.7 ± 1.1	179.1 ± 13.3
Infected (- BER)	43.23 ± 1.7 [a]	30.81 ± 1.0 [a]	159.3 ± 2.5 [a]	671.4 ± 35.1[a]
Non-infected (+ BER)	25.73 ± 1.3 [a]	27.94 ± 1.7 [a]	161.4 ± 4.5 [a]	6146.8 ± 223.9 [a]
Infected (+ BER)	30.74 ± 1.1 [b]	24.57 ± 1.4 [b]	175.6 ± 2.0 [a b c]	8317.7 ± 103.8 [a b c]

Values are means ± SE. a: Significant against non-infected (-BER) group at $P \leq 0.05$, b: Significant against infected (-BER) group at $P \leq 0.05$, c: Significant against non-infected (+BER) group at $P \leq 0.05$.

NE, DA, 5-HT and 5-HIAA contents. On the other hand, BER administration induced a highly significant increment in Na^{+} and Cl^{-} ions contents. Moreover, a partial recovery was observed in Ca^{2+} and Mg^{2+} ions contents in brain tissue (Peng et al., 2004). Shin et al., (2000) showed that BER *in vitro* exerts an inhibitory effect on catecholamine biosynthesis, e.g. on DA synthesis in neuronal cells. Moreover, it has been suggested that BER is an antagonist of brain DA receptors (Huang and Jin, 1992). In addition, Peng et al. (2004) reported that BER at 100 mg/kg after acute treatment decreased the levels of monoamines and increased their turnover rates and possessed anxiolytic-like activity. The anxiolytic mechanism of BER might be related to the decrease in 5-HTergic system activity by activating somatodendritic 5-HT_{1A} autoreceptors and inhibiting postsynaptic 5-HT_{1A} and 5-HT_2 receptors. This may be explaining the reduction in 5-HT and 5-HIAA contents. Kulkarni and Dhir, (2008) concluded that BER influenced either DAergic system by monoamine oxidase-B inhibiting property or by blocking the reuptake of DA by inhibiting its transporter. Moreover, it has been reported that BER has potent inhibitory effects against Ca^{2+} influx via NMDA-receptor on hippocampal pyramidal cells (Yoo et al., 2006). Hence, BER was known as an antidepressant substance. Lau et al. (2001) and Nicholson et al. (2002) showed that in neuronal tissues, the tricyclic antidepressant drug has been shown to inhibit Ca^{2+}-activated K^{+} channels. These may be explaining the decline in Ca^{2+} ion contents in mice brain tissue in the present study. Moreover, Mg^{+2} depletion in mice produce an increase in anxiety and depression-like behavior (Singewald et al., 2004). BER was known as an anxiolytic and antidepressant, so this may explain the increment of Mg^{2+} ion content in mice brain tissue in this study. In addition, electrophysiological studies have demonstrated that BER prolonged the action potential duration and effective refractory period in Purkinje fibers (Dai, 2000). Na^{+} channels are present at distinct sites in neurons, where they sub-serve different functions and play distinct roles.

Some Na^+ channels, especially those at the initial axon segment, initiate action potentials and control firing thresholds. Postsynaptic somatodendritic Na^+ channels act in concert with a range of ligand-gated and voltage gated channels to generate neuronal discharges. In contrast presynaptic Na^+ channels contribute to the regulation of neurotransmitter release (Prica et al., 2008).

Collectively, BER increase the release of the neurotransmitters and consequently, decrease the contents of monoamines and could be considered as a neuro-modulator of *S. mansoni*-infected mice brain.

ACKNOWLEDGMENTS

This work was supported by the distinguished scientist fellowship program, King Saud University, Saudi Arabia.

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African Journal of Pharmacy and Pharmacology Vol. 7(7), pp. 375-388, 22 February, 2013
Available online at http://www.academicjournals.org/AJPP
DOI: 10.5897/AJPP12.1246
ISSN 1996-0816 ©2013 Academic Journals

Full Length Research Paper

Physiochemical, minerals, phytochemical contents, antimicrobial activities evaluation and fourier transform infrared (FTIR) analysis of *Hippophae rhamnoides L.* leaves extracts

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Accepted 14 January, 2013

The present research study was designed to characterize the physiochemical, minerals, phytochemicals, antimicrobial activities and FTIR spectra analysis of various extracts of *Hippophae rhamnoides L.* leaves. The physiochemical and mineral content of *H. rhamnoides L.* leaves revealed that fiber was 18.0 ± 2.64%, protein was 10.45 ± 0.88% and carbohydrate value calculated was found to be 68.75 ± 1%. Sodium was 3,000 ± 1 ppm, calcium was 7,800 ± 1 ppm and potassium was 6,200 ± 2 ppm, referred to as a high concentration while qualitative phytochemicals investigation showed that tannins, phenols and flavonoids were present in large quantity. The highest antibacterial zone of inhibition was observed in aqueous and methanolic extracts against *Staphylococcus aureus* and *Vibrio cholerae* (21 ± 1 mm), and the lowest zone of inhibition measured 07 ± 0 mm against *V. cholerae* (ethyl acetate extracts). The antibacterial minimum inhibitory concentration values of extracts were determined, ranging between 40 to 120 mg/ml, and minimum bactericidal concentration values of the extracts ranged between 50 and 135 mg/ml. The highest antifungal zone of inhibition was calculated against *Alternaria alternata* (18 ± 0 mm) followed by *Aspergillus parasiticus* (17 ± 1 mm) of methanolic extract, while *A. alternata* (07 ± 1 mm) and *Penicillium digitatum* (07 ± 0 mm) were the least, found in chloroform and aqueous extract, respectively. Further assessment of antifungal minimum inhibitory concentration and minimum fungicidal concentration ranged between 40 to 135 and 50 to 180 mg/ml, respectively. The Fourier transform infrared (FTIR) spectra of all extracts revealed the presence of different functional groups ranging from hydroxyl (OH) stretching for hydroxyl group, alkanes (C-H), alkenes (C=C), aromatic rings (C=O), carboxylic (C=O) and amides (aromatic).

Key words: Seabuckthorn leaves, antibacterial, antifungal, secondary metabolites, *Hippophae rhamnoides L.* leaves, functional groups.

INTRODUCTION

Medicinal plants are abundantly available all over the world and are more focused than ever because they have the ability to produce many benefits to human society, especially for the treatment of various types of diseases.

According to World Health Organization (WHO), more than 80% of the world's population relies on conventional medicines for various types of ailment. The therapeutic properties of plants are present in some chemical compounds that cause a specific physiological action in the human body. The most important of these bioactive compounds of plants are alkaloids, flavonoids, tannins and phenolic compounds (Duraipandiyan et al., 2006). The alkaloids have antimicrobial, anticancer, cytotoxic

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and antimalarial properties while flavonoids possess high antibacterial activity and are more effective in the treatment of inflammation, allergy, cancer, viral infection and hypertension. Tannin has shown high activities against viral and bacterial infection as well as act as strong antioxidant (Maryam et al., 2012).

The phytochemical research based on ethno-pharmacological information is generally considered an effective approach in the discovery of new anti-infective agents from higher plants (Duraipandiyan et al., 2006). Worldwide generally and in the third world countries particularly, the infectious diseases (bacterial) are responsible for many human deaths (Nathan, 2004). Bacterial microorganism such as *Pseudomonas*, *Bacillus*, *Staphylococcus* and *Salmonella* are the major pathogens which are responsible for many live threatening diseases. These human bacterial pathogens have the capacity and ability to live in unsuitable environment due to their poly habitats in the earth (Ahameethunisa and Hoper, 2010). Pathogenic fungi are responsible for many different types of diseases due to subcutaneous, deep inside and superficial infections. Humans infected with pathogenic fungi have increased drastically in all parts of the world. Human systemic infections is caused by *Candida albicans, Aspergillus flavus and A. niger,* which are found throughout the world and caused different types of diseases such as candidiasis, aspergillosis etc. (Gutal, 2011). So there is a need to develop new antibiotics from natural sources, especially from plants, which do not exhibit many of the side effects always associated with the synthetic antibiotics. A normal human diet consists of water, vitamins, fats, proteins, carbohydrates and minerals. Human health always depends on balanced mineral contents in the body. If the balance is disturbed (below or above the limits), it results in the abnormalities of human health, so balanced mineral diet play important role in relation to human health (Archa et al., 2010).

Hippophae rhamnoides L. commonly known as sea-buckthorn is a shrub belonging to the family Elaeagnaceae in which male and female plants are different (unisexual). The leaves of seabuckthorn are commonly green on the upper side while green silver ash color on lower side, with a rough and thick bark (Raj et al., 2011). The whole plant, particularly leaves and berry, are considered a good source of vitamins (C, E, A and K) and other important vital compounds (flavonoids, sterols and carotenoids), which possess highest medicinal and nutritional properties (Chen et al., 1991; Yang and Kallio, 2001). The seabuckthorn plant has been used tradition-ally as nutritional supplement, medicine, fuel and as fence. The plant has been extensively used for treatment of gastrointestinal tract (GIT) disorder, ulcer, hepatic disorder, thrombosis, cancer, tendon and ligament injuries (Tsering et al., 2010).

Sea-buckthorn spp *turkestanica* seed elemental analy-sis revealed that it has Na, K, P, Mg, Ca, Zn, Fe and Ag (Alam and Ijaz, 2009). The stem and root extracts of *H. rhamnoides* showed strong antibacterial and antifungal activities (Jong et al., 2010). Sanjay et al. (2011) studied the antimicrobial activity of seed extract and leaf crude extract and seed oil of sea-buckthorn (*H. salicifolia* D. Don) and found that fresh leaves [supernatant concentrated and supernatant dried extract dissolved in Dimethyl sulfoxide (DMSO)] and seed extract have antibacterial activity against gram +ve bacteria. While gram -ve bacteria showed resistance against these extracts except *Agrobacterium tumefaciens.* Only seed extract exhibited antifungal activity against *Tilletia and Mucor*, whereas no activity was observed in case of *Rhizopus.*

Keeping in view the importance of sea-buckthorn, the present study was aimed to evaluate sea-buckthorn leaves extracts for its physiochemical, minerals, phytochemicals content, antimicrobial activities and Fourier transform infrared (FTIR) spectra profiling analysis.

MATERIALS AND METHODS

Sample collection

The fully grown healthy sea-buckthorn leaves (SBL) were collected from Pakistan Council of Scientific and Industrial Research (PCSIR) Skardu, Pakistan. The leaves were slightly washed to remove any dust, and dried in shade. The dried leaves were then converted into powder and transferred into air tight plastic bags until they were used.

Leaves extraction

Fifty grams powder of sea-buckthorn leaves were taken and extracted in 250 ml of aqueous ethanol, acetone, methanol, ethyl acetate, chloroform and n-hexane for 48 h. These extracts were then filtered under vacuum through Whatman filter paper (No.1) into a Buchner flask. The extracts were concentrated in a rotary evaporator and then transferred into a sterilized beaker for heating in a water bath at 50°C to obtain a dry residue. The resultant crude extracts were transferred into airtight sample bottles and kept at 4°C until they were used.

Physiochemical analysis of leaves

The SBL powder used for physiochemical analysis, which included parameters like moisture, ash, fat, pH, total suspended solids (TSS), acidity, crude fiber, nitrogen, protein, total sugar, reducing sugar and non-reducing sugar. They were determined in accordance with standard procedures (Association of Official Analytical Chemists (AOAC), 2003). Percentage carbohydrate value was calculated by the given method (Indrayan et al., 2005).

Mineral analysis of leaves

Elemental contents were determined by wet digestion procedure. Minerals like sodium and potassium were determined with the help of Flame Photometer (Jenway PFP7). Heavy and alkaline metals like Ca, Mg, Fe, Al, Mn, Zn, Si, Ba, Cd, Pb, Cr and Ni, were determined by Atomic absorption spectrophotometer (Hitachi Zeeman Japan Z-8000) according to the standard methods (AOAC, 2003).

Phytochemical qualitative screening

Phytochemicals were analyzed qualitatively using the method as described by Harborne (1998).

Antimicrobial activities

Tested microorganism

Bacterial cultures of *Staphylococcus aureus, Escherichia coli, Enterococcus faecalis, Klebsiella pneumoniae, Pseudomonas aeruginosa, Vibrio cholerae, Salmonella typhi, Bacillus cereus, B. subtilis, Citrobacter freundii* and pure cultures of *A. niger, A. parasiticus, A. flavus, A. fumigatus, A. oryzae, Fusarium oxysporum, Rhizopus arrhizus, Alternaria alternata, C. albicans and Penicillium digitatum* of fungi were obtained from Food Microbiology Laboratory of PCSIR Laboratories Complex, Jamrude Road, Peshawar Khyber Pakhtunkhwa (KPK), Pakistan, and used in the present study. These cultures were maintained on slants of nutrient agar (bacteria) and potato dextrose agar (fungi) and kept in a refrigerator to subculture after every week.

Antibacterial activity determination

Antibcterial activity was determined using well agar diffusion method (El-mahmood, 2009) with minor modifications. One milliliter (1 ml) of tested bacterial strains adjusted to 0.5 Mac Farland units (1.0×10^8 cfu/ml) was inoculated into plate. The molten nutrient agar at 45°C was added into each plate and gently shaken to ensure uniform mixing of the media and culture. Wells were punched in the solidified agar with the help of a sterile 6 mm cork borer. The dried SBL extracts, 1000 mg, were dissolved in 5 ml of DMSO. Fifty microliter (50 µl) of the 200 mg/ml of each extracts was pipetted into each well. Fifty microliter of each of the dimethyl sulfoxide (negative control) and Ciproxin (0.5 mg/ml) solution as positive control was used. The plates were incubated at 37°C for 18 h. The antibacterial activity was determined by measuring the diameters of zones of inhibition.

Determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

The broth dilution method was carried out to determine the minimum inhibitory concentration. The sea-buckthorn leaves extracts were diluted to various concentrations ranging from 20 to 200 mg/ml in nutrient broth. Five hundred microliter (500 µl) of each concentration was added to sterile nutrient broth (2 ml) in test tubes placed on a test tube rack. Then, 1 ml (1×10^8 cfu/ml) of bacterial culture of the respective strain was added to the content of the test tubes and incubated at 37°C for 18 h, Ciproxin solution with different concentration range from 0.10 to 0.5 mg/ml was used as positive control. The lowest concentration of the testing material that did not allow any visible growth against experimental bacteria was taken as MIC. The MBC was determined by pipetting 100 µl of culture from each of the broth tubes having no growth, and introduced into fresh agar plates. The plates were incubated for 48 h and then observed for growth. The concentration of the extracts/control without visible growth was calculated as the MBC (EL-mahmood, 2009).

Antifungal activity

Antifungal activities were assessed according to the method of Ogu et al. (2011) with minor modifications. One ml of each standardized spore suspension (10^5 spores/ml) was spread on the surface of the *Sabouraud dextrose agar* (SDA) plates. Then, sterile cork borer (6 mm in diameter) was used to make a well at the centre of each inoculated/cultured plates. SBL extracts of 1,000 mg were dissolved in 5 ml of DMSO (200 mg/ml), and 50 µl of each extract was applied into each respective well. 50 µl each of pure dimethyl sulfoxide and fluconazole (0.5 mg/ml) were used as negative and positive control, respectively. The plates were kept in the incubator for 1 to 7 days at ambient temperature and then observed for anti-fungal activities.

Determination of minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC)

The MIC of the extracts was carried out using broth dilution method of Ogu et al. (2011), with minor modification. Serial dilutions of each extracts in the broth were made to obtain the concentration in the range of 20 to 200 mg/ml (extracts) and 0.1 to 0.5 mg/ml (fluconazole). One milliliter (1 ml) standardized inoculums (10^5 spores/ml) were inoculated into each broth having different extract concentrations and then incubated for 1 to 7 days at 30°C to observe turbidity. MIC was calculated on the basis of no turbidity observation of lowest concentration in the test tubes. For MFC determination, the contents of the MIC in the serial dilution were then sub-cultured on the media and incubated at 30°C for one to seven days and observed for colony growth. The MFC was the plate with the lowest concentration of extracts and without colony growth.

Fourier transforms infrared spectroscopy (FTIR) analysis of SBL extracts

Fourier transform infrared (FTIR) technique was used for the identification of different functional groups in each extract. The infrared spectroscopy (IR) spectrum was obtained using FTIR Prestige -21 Shimadzu Japan. The sample was scanned from 3,900 to 500 cm^{-1}, operating at a resolution of 4 cm^{-1}, with 10 number of scans.

Statistical analysis

Statistical package for social sciences (SPSS) version 14 was used to analyze the data. The data were presented in the form of mean ± standard deviation (SD).

RESULTS

Extractive values, physiochemical and mineral analysis

The physiochemical analyses of SBL are shown in Figure 1. The moisture (%) content was 8.0 ± 1, ash (%) content was 7.0 ± 0.3, fat (%) value was 5.8 ± 0.4, pH value was 4.0 ± 0.2, TSS was 1.0 ± 0.25%, acidity was 0.60 ± 0.17%, fiber was 18.0 ± 2.64%, total sugar was 0.8 ± 0.01%, reducing sugar was 0.7 ± 0.01%, non-reducing sugar was 0.1 ± 0.01%, nitrogen was 1.82 ± 0.24% and protein was 10.45 ± 0.88%. The carbohydrate value calculated in sea-buckthorn leaves was found to be 68.75 ± 1%. The mineral content of seabuckthorn leaves are shown in Figure 2. Sodium was found in highest concen-

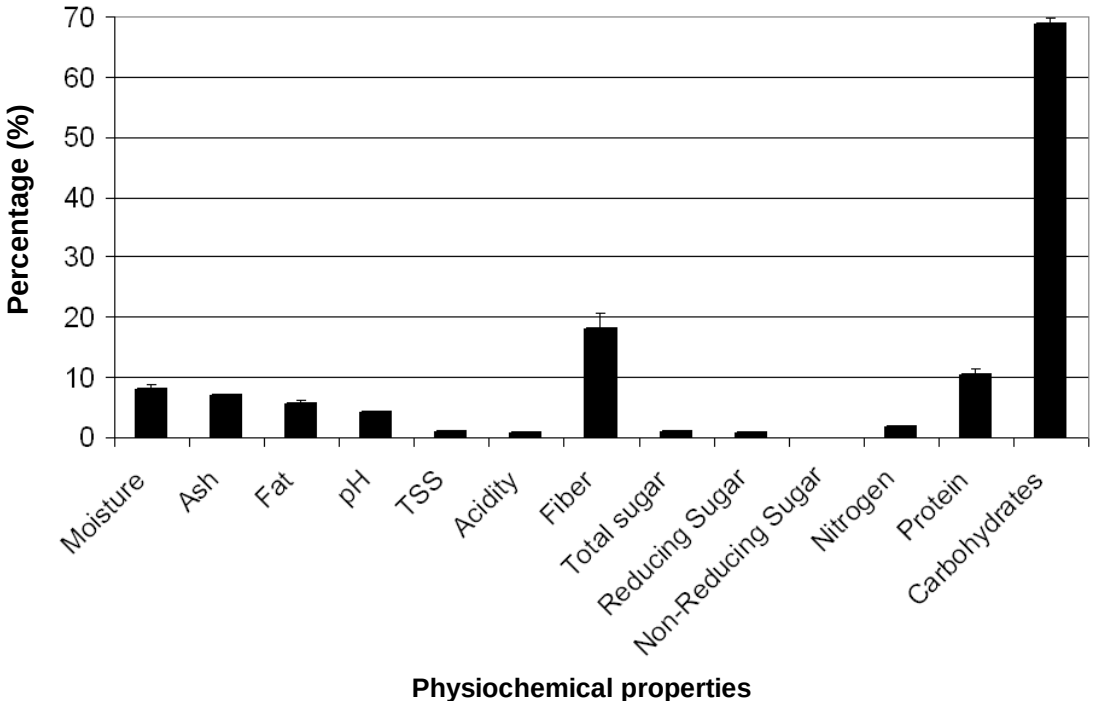

Figure 1. Physiochemical analysis of *Hippophae rhamnoides L.* leaves extracts.

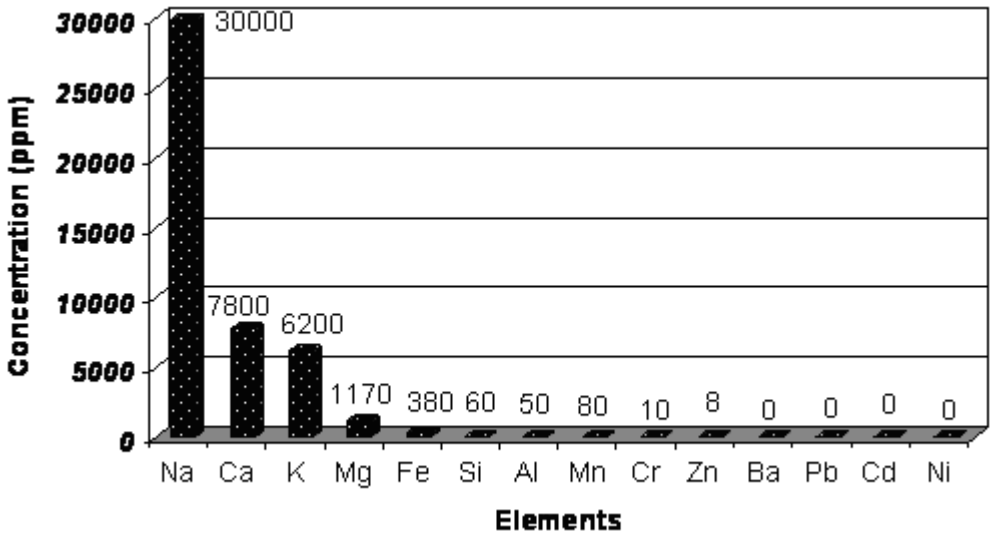

Figure 2. Minerals analysis of *Hippophae rhamnoides L.* leaves extracts.

tration (30,000 ± 1 ppm) and zinc was found in lowest concentration (8 ± 1 ppm), while the reaming elements were calculated (ppm) in the order Ca > K > Mg > Fe > Si > Al > Mn > Cr. Barium, cadmium, lead and nickel were not detected in seabuckthorn leaves. Extractive values of sea-buckthorn leaves are presented in Figure 3. Acetone showed the highest yield (22.95%) followed by methanol > ethanol > aqueous > chloroform > ethyl acetate > n-hexane.

Phytochemicals

As can be seen from Table 1, the content of phenols and flavonoids were found high while flavones, tannins and amino acids detected in the aqueous extract were in the average range. The terpenoids, glycosides, alkaloids and saponins were not present, and the quantity of steroids noted was very low in the aqueous extract of seabuckthorn leaves. In the methanolic extract of sea-

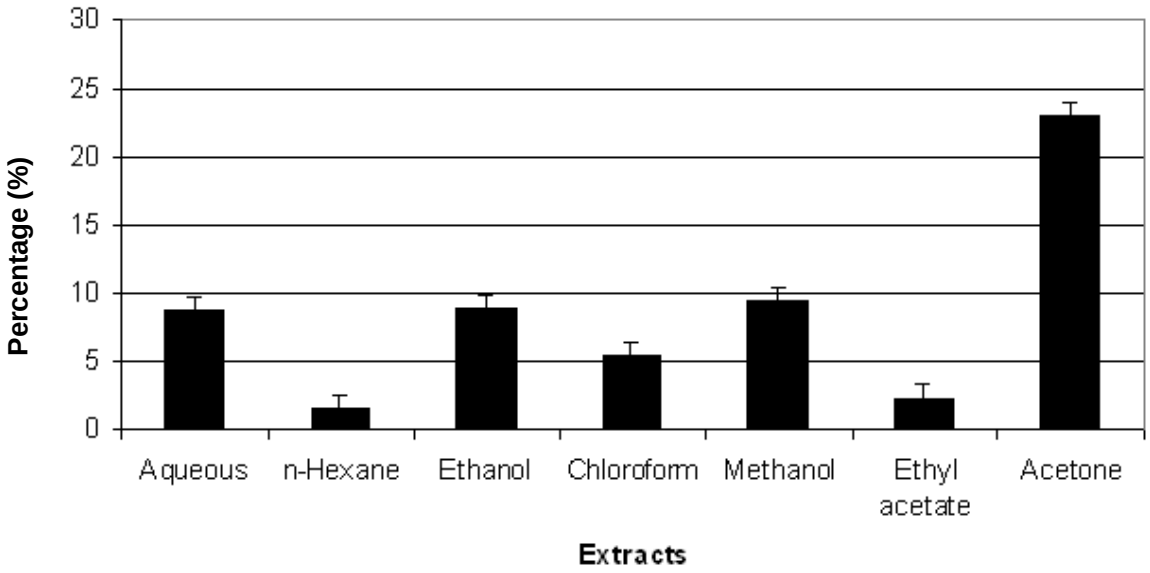

Figure 3. Extractive values of *Hippophae rhamnoides L.* leaves extracts.

buckthorn leaves, phenols, flavonoids, flavones and tannins were present in high concentration followed by terpenoids and amino acids, while saponins were not found in the methanolic extract. The Table 1 data shows that phenols, flavonoids and tannins concentrations were recorded maximum, while the least contents of terpenoids, steroids glycosides and alkaloids were noted in the ethanolic extract. Glycosides, flavones and saponins were absent in the ethyl acetate extract while average amount of flavonoids and tannins were noted in acetone extract. The minimum concentration of steroids, alkaloid, flavonoids and amino acids were present in the chloroform extract. Average contents of terpenoids were recorded in hexane extract of SBL, while the concentration of steroids, alkaloids and tannin were found in low range.

Antimicrobial activities

The mean zones of inhibition of different extract against 10 bacterial species are summarized in Table 2. The aqueous extract of sea-buckthorn leaves were found more active against *S. aureus* with 21 ± 1 mm zone of inhibition followed by 20 ± 2 mm against *E. coli,* while the lowest value 11 ± 0 mm was recorded for *S. typhi*. The methanolic extract showed the highest value of 21 ± 1 mm against *V. cholerae* while 19 ± 1 mm zone of inhibition was noted against *E. coli*. The zone of inhibition 16 ± 1 mm was measured against both *B. cereus* and *C. freundii* while the methanolic extract was found less active and showed 09 ± 0 mm zone of inhibition against *S. typhi*. The highest zone (20 ± 1 mm) of inhibition was recorded against *P. aeruginosa*, while *S. typhi* were found more resistant against the ethanolic extract, with 08 ± 0 mm zone of inhibition. The ethyl acetate extract was found more resistant against both *P. aeruginosa* and *B. cereus*, with 15 ± 1 mm zone of inhibition, and the least value 10 ± 0 mm zone of inhibition was recorded against *E. coli* by acetone extract. The chloroform extract zone of inhibition values were found in the range of 08 to 16 mm, while all the tested bacteria showed strong resistance against the hexane extract. Ciproxin was used as standard, ranging the value of zone of inhibition from 20 to 28 mm.

Table 3 data shows the MIC and MBC of the different extracts of SBL. The aqueous extract of SBL was found more active against S. *aureus*, with MIC and MBC values of 40 and 60 mg/ml, respectively. *P. aeruginosa* showed high resistance, with 100 and 110 mg/ml of MIC and MBC, respectively against the methanolic extract while 40 mg/ml MIC and 60 mg/ml MBC showed the highest activity of the ethanolic extract against *E. coli*. The MIC of the ethyl acetate extract were in the range of 65 to 120 mg/ml. MBC of the acetone extract ranged from 85 to 135 mg/ml, and the lowest MIC of the chloroform extract, 70 mg/ml, was recorded against *K. pneumoniae*.

The antifungal activity of the aqueous extract was observed, with maximum 16 ± 1 mm zone of inhibition against *F. oxysporum* and the minimum value 9 ± 0 mm was observed in methanolic extract against *A. oryzae*. Ethanolic extract showed the highest 14 ± 1 mm zone of inhibition against both *A. niger* and *C. albicans*, while ethyl acetate extract was found more active, with 15 ± 0 mm zone of inhibition against *R. arrhizus*. Extract of acetone and chloroform showed the same value (10 ± 1 mm) of zone of inhibition against *C. albicans* and the n-hexane extract was found inactive against all the tested

Table 1. Phytochemical analysis of *Hippophae rhamnoides L.* leaves extracts.

Chemical constituents	Aqueous	Methanol	Ethanol	Ethyl acetate	Acetone	Chloroform	n-Hexane
Terpenoids	ND	+ +	+	++	+	+ +	+ +
Steroids	+	+	+	+	+	+	+
Glycosides	ND	+	+	ND	+	ND	ND
Alkaloids	ND	+	+	+	+	+	+
Phenols	+ + +	+ + +	+ + +	+ +	+	+ +	ND
Flavonoids	+ + +	+ + +	+ + +	+ +	+ +	+	ND
Flavones	+ +	+ + +	+ +	ND	ND	ND	ND
Tannins	+ +	+ + +	+ + +	+ + +	+ +	+ +	+
Saponins	ND	ND	ND	ND	ND	ND	ND
Amino Acids	+ +	+ +	+ +	+	+	+	ND

+ sign shows detection level of the phytochemicals present in extracts. + = small quantity, + + = average quantity, + + + = large quantity, ND = not detected.

Table 2. Antibacterial activities of *Hippophae rhamnoides L*. leaves extracts.

Tested bacteria	Zone of Inhibition (mm)								
	Aqueous	Methanol	Ethanol	Ethyl acetate	Acetone	Chloroform	n-Hexane	C^+	C^-
S. aureus	21±1	14±1.7	13±0	12±1	11±1	11±0	0.0	28±1	0.0
E. coli	20±2	19±1	18±1	13±1	10±0	16±0	0.0	26±1	0.0
E. faecalis	15±1.7	14±1.7	16±0	08±0	16±1	13±0	0.0	24±0	0.0
K. pneumoniae	14±1	17±2	17±1	12±1	13±0	11±0	0.0	25±1	0.0
P. aeruginosa	14±1	11±1.7	20±1	15±1	18±0	08±0	0.0	23±0	0.0
V. cholerae	13±0	21±1	14±0	07±0	16±0	09±1	0.0	24±0	0.0
S. typhi	11±0	09±0	08±0	10±0	17±0	10±1	0.0	22±0	0.0
B. cereus	14±1	16±1	13±1	15±1	11±0	13±1	0.0	25±1	0.0
B. subtilis	12±1	14±0	15±0	09±0	12±1	12±0	0.0	23±0	0.0
C. freundii	13±1	16±1	15±1	10±1	16±0	11±0	0.0	20±1	0.0

C^+ = Positive Control (Ciproxin 0.5 mg/ml), C^- = Negative Control (DMSO), each value represents mean ± standard deviation (SD) (n = 3). 0 = no zone of inhibition.

fungi. Fluconazole was used as a standard antifungal agent, showing the values of zone of inhibition from 16 to 24 mm (Table 4).

The aqueous extract of SBL was found highly active against *F. oxysporum*, with 50 mg/ml MIC value and 65 mg/ml MFC value (Table 5). The maximum (90 mg/ml) MIC value of the methanolic extract was observed against *A. oryzae*, while the least value (90 mg/ml) of MFC of ethanolic extract was noted against *P. digitatum*. Ethyl acetate and acetone extracts of SBL showed poor activity as reflected by the high MIC and MFC values as compared to methanol, ethanol and aqueous extracts. The MFC of the chloroform extract of SBL were in the range of 100 to 150 mg/ml.

FTIR analysis

The FTIR spectra analysis was utilized to identify the functional group of the active ingredients on the basis of peak value in the vicinity of infrared radiation. The results of FTIR peak values and functional groups of SBL aqueous extract are represented in Figure 4. IR-spectrum shows strong absorption peaks at 3242.34 (broad), 2929.87 (small short) and 1593.20 cm^{-1} (sharp), which correspond to the presence of hydroxyl (OH), alkane (C-H) and aromatic rings (C=O) functional groups, respectively. SBL methanol extract (Figure 5) showed peak values 2922.16, 2852.72, 1693.50 and 1600.92 cm^{-1} which could be attributed to the existence of functional groups alkanes (sharp peak)**,** alkanes, carboxylic and amides, respectively. Ethanol extracts (Figure 6) showed broad peak values (3315.63 cm^{-1}) of OH groups, sharp and strong peak (2924.09 cm^{-1}) of alkane, small peak (2854.65 cm^{-1}) for alkane, and small and weak peaks of alkene (1735.93 and 1604.77 cm^{-1}). Ethyl acetate peak (cm^{-1}) data are shown in (Figure 7) and confirm the presence of hydroxyl, alkane and carboxyl group.

Table 3. Antibacterial MIC and MBC of *Hippophae rhamnoides L.* leaves extracts.

Tested bacteria	MIC/MBC	Concentration (mg/ml)						
		Aqueous	Methanol	Ethanol	Ethyl acetate	Acetone	Chloroform	Ciproxin
S. aureus	MIC	40.0	60.0	55.0	70.0	60.0	80.0	0.10
	MBC	60.0	80.0	70.0	80.0	95.0	100.0	0.10
E. coli	MIC	65.0	55.0	40.0	120.0	60.0	85.0	0.10
	MBC	75.0	70.0	60.0	125.0	90.0	90.0	0.20
E. faecalis	MIC	70.0	60.0	45.0	100.0	115.0	75.0	0.20
	MBC	80.0	80.0	66.0	120.0	135.0	90.0	0.20
K. pneumoniae	MIC	80.0	55.0	70.0	65.0	80.0	70.0	0.20
	MBC	90.0	70.0	80.0	75.0	110.0	90.0	0.20
P. aeruginosa	MIC	80.0	100.0	65.0	70.0	65.0	85.0	0.20
	MBC	85.0	110.0	75.0	125.0	85.0	100.0	0.20
V. cholerae	MIC	90.0	50.0	75.0	110.0	70.0	85.0	0.20
	MBC	95.0	60.0	80.0	130.0	90.0	110.0	0.20
S. typhi	MIC	90.0	50.0	55.0	65.0	100	85.0	0.22
	MBC	100.0	55.0	75.0	75.0	130	125.0	0.24
B. cereus	MIC	75.0	55.0	70.0	80.0	75.0	85.0	0.20
	MBC	80.0	70.0	70.0	120.0	85.0	100.0	0.20
B. subtilis	MIC	80.0	60.0	65.0	70.0	80.0	80.0	0.20
	MBC	90.0	90.0	80.0	85.0	120.0	95.0	0.25
C. freundii	MIC	80.0	50.0	50.0	90.0	90.0	85.0	0.22
	MBC	110.0	70.0	70.0	125.0	110.0	100.0	0.25

Each value represents mean (n = 3).

Acetone extract FTIR spectra (Figure 8) peak values were 3350.35 (OH group), 2924.09 cm^{-1} (CH3-CH2 alkane stretch), 2852.72 cm^{-1} (alkane) and C=O group (1703.14, 1693.50 cm^{-1}). Four considerable peaks were observed in chloroform (Figure 9) and found as an OH, alkane and (C=O) functional groups. Two sharp and strong peaks (2922.16 and 2850.79 cm^{-1}) attributed the existence of alkane group in n-hexane (Figure 10) extract and another two peaks values seen in the region of 3398.57 and 1716.65 cm^{-1} were OH and C=O groups.

DISCUSSION

Plants are always known to provide shelter, food, medicine etc. to human beings and other living organisms. Medicinal plants hold some pharmacologically active values which are used from the ancient times and were exploited in phyto drugs for the cure of different types of diseases.

The physiochemical analysis indicated that sea-buckthorn leaves are very rich source of protein. Due to high protein content of fiber and protein, it can be used as animal feed, and its purified form is also used for human consumption. In sea-buckthorn leaves, protein is one of the important components (chemically) that has an important nutritional value in animal feed and can be used as a source of unconventional protein for human food (Pirie, 1986). The results (Figure 1) showed 10.45/100 g proteins in the sea-buckthorn leaves while *H. rhamnoides* subsp. sinensis leaves were reported to contain an average of 17.1/100 and 16.2/100 g protein in dried leaf (Li and Wardle, 2003). Sea-buckthorn seeds moisture content was 5.5% and ash content was 2.05% (Alam and Ijaz, 2009), which revealed that in our findings, the leaves have high moisture and ash content (Figure 1). Similarly, total sugar content in sea-buckthorn pulp was 2.86% (Tsering et al., 2010), which was very high as

Table 4. Antifungal activities of *Hippophae rhamnoides L.* leaves extracts.

Tested fungus	Zone of inhibition (mm)								
	Aqueous	Methanol	Ethanol	Ethyl acetate	Acetone	Chloroform	n-Hexane	C^-	C^+
A. niger	13±1	15±1	14±1	10±1	08±0	12±0	0.0	0.0	18±0
A. parasiticus	14±0	17±1	12±1	09±0	09±0	11±1	0.0	0.0	22±0
A. flavus	13±1	16±1	10±1	08±1	10±0	09±0	0.0	0.0	20±0
A. fumigatus	09±0	13±0	11±0	11±1	09±0	08±1	0.0	0.0	23±0
A. oryzae	12±0	09±1	11±1	12±0	08±0	09±1	0.0	0.0	24±0
F. oxysporum	16±1	13±0	13±0	13±1	12±1	10±0	0.0	0.0	22±0
R. arrhizus	15±0	14±0	09±0	15±0	11±0	8±0	0.0	0.0	16±0
A. alternata	11±1	18±0	10±0	11±1	09±0	07±1	0.0	0.0	19±0
C. albicans	12±1	14±1	14±1	09±0	10±1	10±1	0.0	0.0	17±0
P. digitatum	07±0	13±1	10±0	08±0	09±1	12±0	0.0	0.0	21±0

C^+ = positive control (Fluconazole 0.5 mg/ml), C^- = negative control (DMSO), each value represents mean ± SD (n = 3). 0 = no zone of inhibition.

Table 5. Antifungal MIC and MFC of *Hippophae rhamnoides L. leaves* extracts.

Tested fungus	MIC/MFC	Concentration mg/ml						
		Aqueous	Methanol	Ethanol	Ethyl acetate	Acetone	Chloroform	C^+
A. niger	MIC	60	50	80	120	120	135	0.35
	MFC	75	80	100	130	145	150	0.40
A. parasiticus	MIC	100	55	90	110	115	95	0.30
	MFC	130	70	100	115	135	105	0.35
A. flavus	MIC	120	85	120	100	100	80	0.35
	MFC	145	90	160	120	140	100	0.40
A. fumigatus	MIC	90	70	100	80	110	100	0.30
	MFC	100	75	115	90	115	115	0.35
A. oryzae	MIC	60	90	130	120	120	110	0.25
	MFC	75	135	140	140	135	115	0.30
F. oxysporum	MIC	50	60	90	100	85	130	0.30
	MFC	65	70	120	120	105	145	0.35
R. arrhizus	MIC	65	55	110	100	105	120	0.50
	MFC	75	85	130	135	115	135	0.50
A. alternata	MIC	80	40	100	120	120	130	0.40
	MFC	100	50	120	130	130	140	0.40
C. albicans	MIC	100	80	80	120	95	95	0.30
	MFC	120	140	95	180	100	105	0.35
P. digitatum	MIC	110	80	80	120	105	100	0.25
	MFC	130	120	90	135	130	150	0.35

Each value represents mean (n = 3).

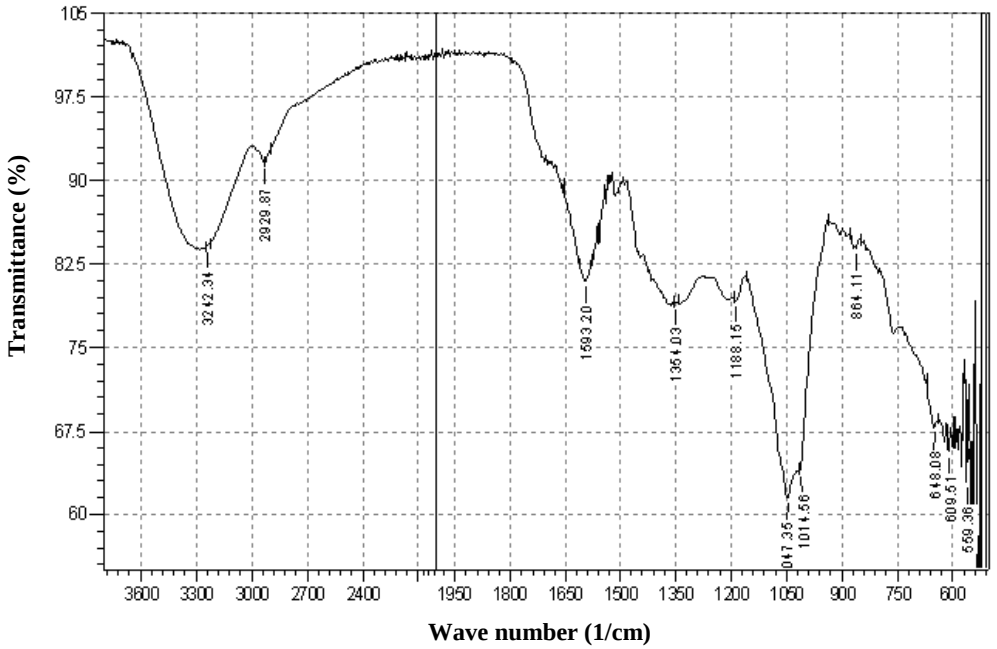

Figure 4. FTIR spectra analysis of aqueous extract of *Hippophae rhamnoides L.* leaves.

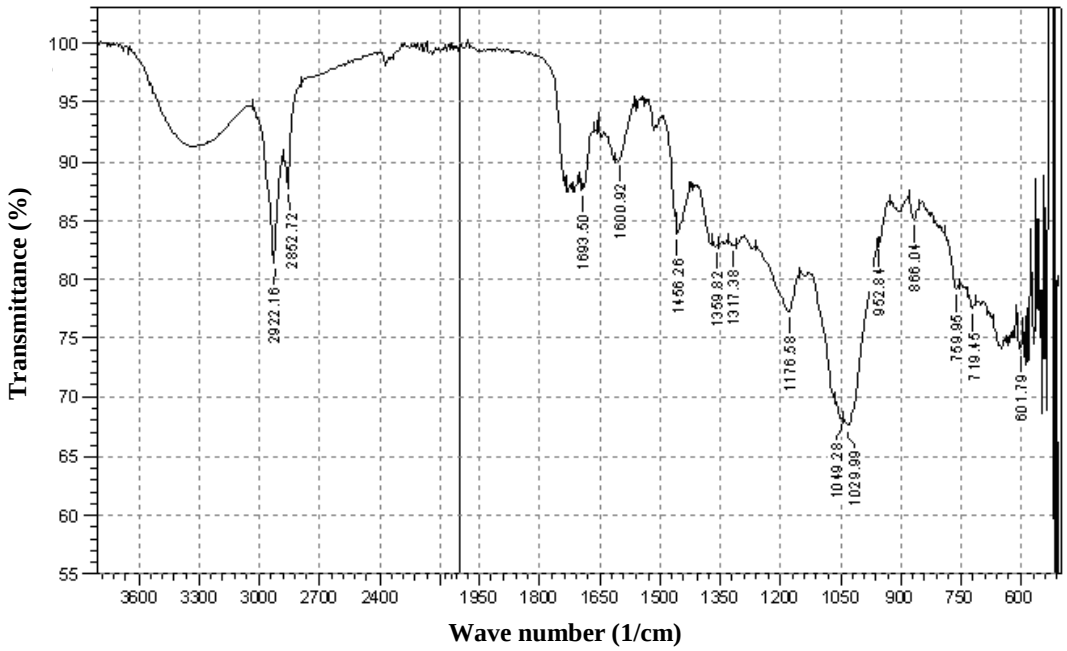

Figure 5. FTIR spectra analysis of methanol extract of *Hippophae rhamnoides L.* leaves.

compared to the sea-buckthorn leaves (Figure 1). Minerals play vital and important role in our body; sodium and potassium normalize acid base balance and osmotic pressure of fluid (body). Similarly, Ca is an important component for the teeth, enzyme (cofactor) and skelton (Okolo et al., 2012). Magnesium and Zn help in the enhancement of muscle regeneration, growth and prevent cardiomyopathy (Chaturvedi et al., 2004).

Previous studies (Alam and Ijaz, 2009) on elemental analysis of sea-buckthorn ssp turkestanica seeds revealed that Na, K, Ca and Fe were found very less as compared to the present study on the seabuckthorn leaves which showed high contents of these elements (Figure 2). In sea-buckthorn pulp, the mineral content

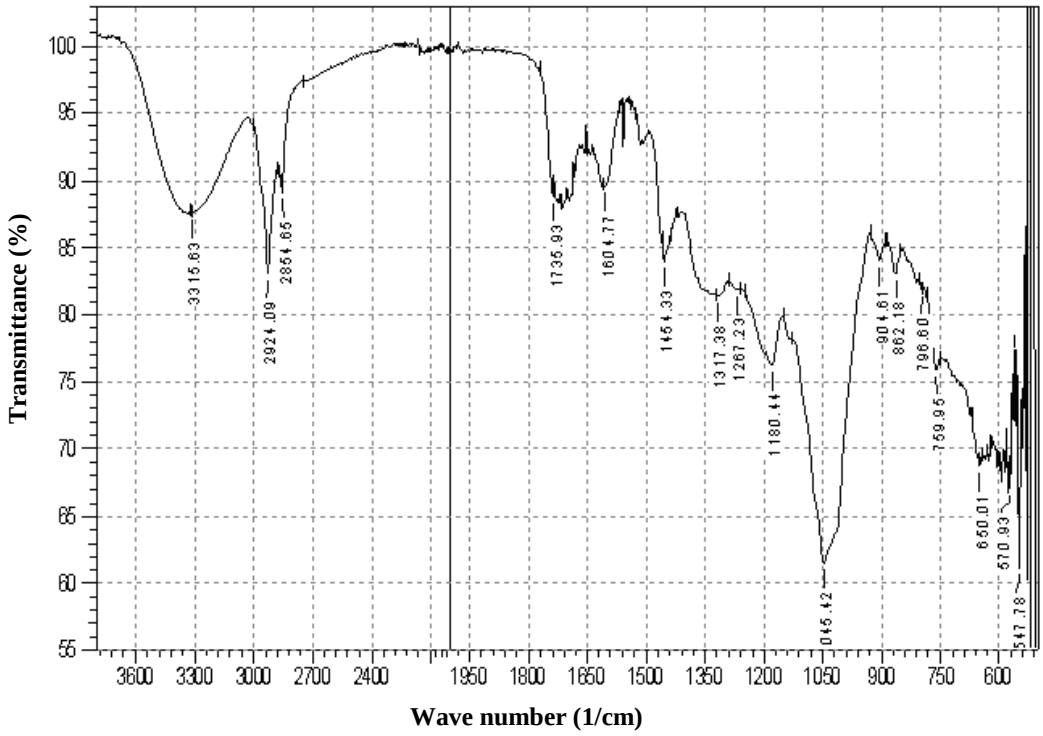

Figure 6. FTIR spectra analysis of ethanol extract of *Hippophae rhamnoides L.* leaves.

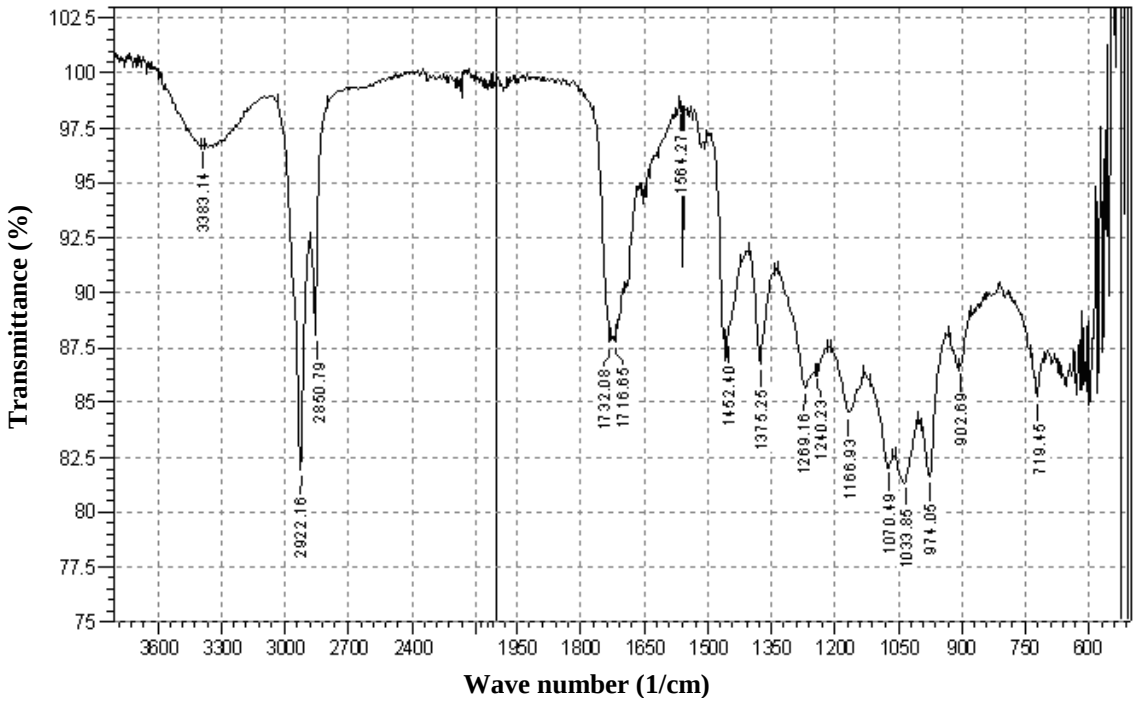

Figure 7. FTIR spectra analysis of ethyl acetate extract of *Hippophae rhamnoides L. leaves*.

(mg/l) of K, Ca, Fe, Mg, Na, Zn and Mn were 647.2, 176.6, 30.9, 22.5, 414.2, 1.4 and 1.06, respectively (Tsering et al., 2010), in sea-buckthorn leaves these minerals values were found high (Figure 2). The present study revealed that the sea-buckthorn leaves are a good source of sodium, potassium and calcium, so it can be

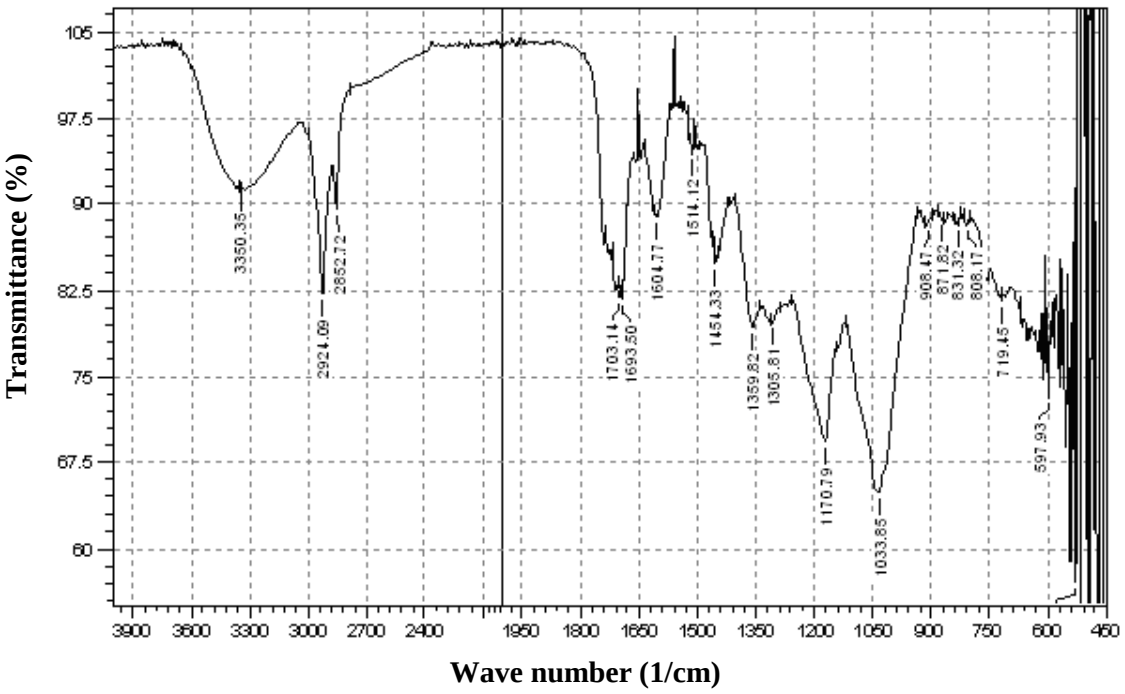

Figure 8. FTIR spectra analysis of acetone extract of *Hippophae rhamnoides L.* leaves.

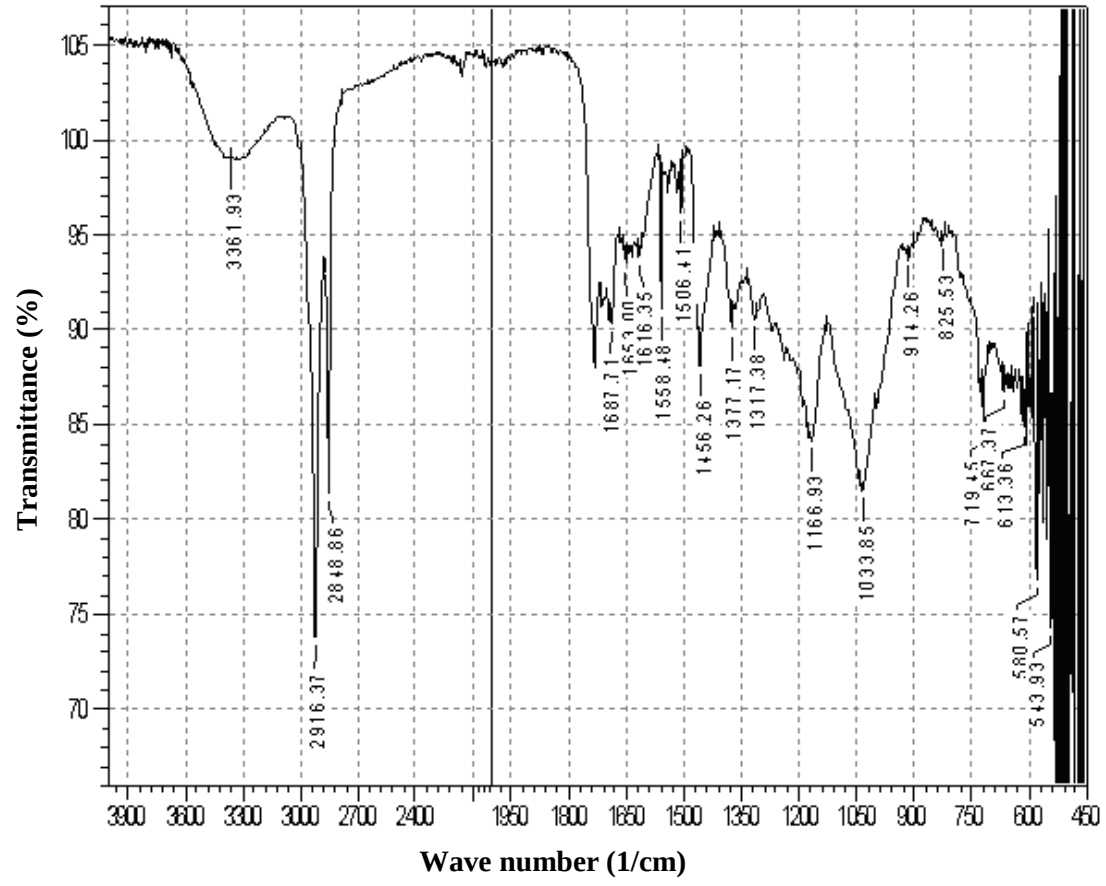

Figure 9. FTIR spectra analysis of chloroform extract of *Hippophae rhamnoides L.* leaves.

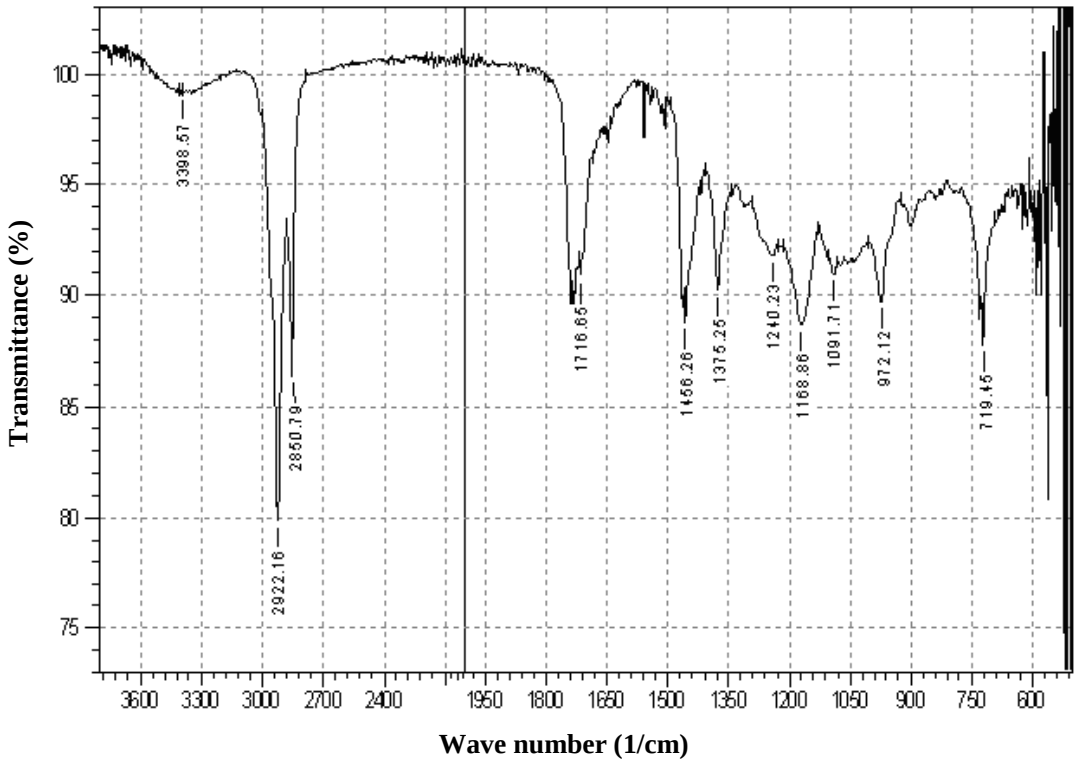

Figure 10. FTIR spectra analysis of n-hexane extract of *Hippophae rhamnoides L*. leaves.

used as animal feed additives as well as for malnutrition cases in human beings.

Phytochemicals

Different types of disease are on the raise globally particularly in the developing countries, and their treatment by synthetic drugs are always associated with many side effects. The use of phytochemical could be a safe way to solve these problems. Phytochemicals commonly produce their antimicrobial activities through special principles which may be different from synthetic drugs (Scalbert, 1991). The tannins containing plants as their main component are astringent in nature and are used for the treatment of stomach disorders such as diarrhea and dysentery (Dharmananda, 2003). Flavonoids are considered as the main phytochemicals present in plant, and are used for the treatment of various types of microbial infections (Xiao, 1980). Alkaloids were absent in methanolic and chloroform + methanolic extracts of sea-buckthorn twigs (Keshab et al., 2010), while present in the leave extracts (Table 1).

Antimicrobials activities

Nowadays, bacterial resistance has increased against a number of valuable antibiotics and it has become essential to search for new sources of antibiotics. Presently, most of the antibiotics available in the market are derived from the natural sources, particularly from different types of plant. The current study has been designed to assess the MIC, MBC and MFC of different extracts of sea-buckthorn leaves against various strains of bacteria and fungi. The susceptibility of the bacteria and fungi to the extracts on the basis of inhibition zones growth varies according to extracting solvent and microorganism. It has been observed that the inhibition zone diameter changed from one another due to variation from plant to plant and organism to organism at diverse concentrations (Mann et al., 2008; El-Mahmood et al., 2008). The hexane extracts however did not exhibit any zone of inhibition against all the test microorganisms; it can be attributed to poor extractive value and absence or low quantity of phytochemicals (Figure 3 and Table 1). Highest activity was verified by the standard antibiotic ciproxin and fluconazole (control). It is not surprising that reference antibiotics have high zone of inhibition, least MIC, MBC and MFC values as compared to the SBL extracts. Because the antibiotic is synthesized by high standard techniques, common plant origin medicines are produced from crude sources which are mostly time exposed to degradation and contamination (El-Mahmood and Amey, 2007).

The MIC values of sea-buckthorn root and stem

extracts against *B. subtilis, S. aureus, E. coli and C. albicans* were reported (Jong et al., 2010) very low as compared to the sea-buckthorn leave extracts (Tables 3 and 5). The sea-buckthorn (*H. salicifolia* D. Don) seed oil, concentrated crude leaf and fresh crude leaf extracts showed no antibacterial and antifungal activity (Sanjay et al., 2011), whereas *H. rhamnoides L*. leave extracts were found active against both bacteria and fungi (Tables 2 to 5). The sea-buckthorn aqueous and methanolic extracts of seeds and pomace showed low zone of inhibition against *B. cereus, S. aureus, E. coli, E. faecalis, K. pneumoniae and P. aeruginosa* reported by Richa et al. (2012), while sea-buckthorn leaves aqueous and methanolic extracts were observed for high zone of inhibition against the studied bacteria. The manifestation of activity against both gram-positive and gram-negative bacteria and fungi is a sign that the plant can be a source of bioactive substance that could be of broad spectrum of activity.

FTIR analysis

FTIR analysis of the SBL extracts shows a strong presence of hydroxyl group which is common in all phenolic compounds. All SBL extracts absorption bands were attributed to (OH) stretching vibrations from phenols, a group of compounds (chemical) containing hydroxyl functional groups (-OH) attached to an aromatic hydrocarbon. Phenolic compounds from natural resources displayed antifungal activity (Soundararajan et al., 2012). The location site(s) and the amount of hydroxyl groups found in the phenols are related to their relative toxicity towards microorganisms, with evidence that increased hydroxylation is directly proportion to toxicity (Geissman, 1963). Also, carboxylic acids were found to be linked with many antimicrobial and antifungal activities which are found to exist in various plant metabolite molecular structures such as ursolic acid, which had been reported as a strong antibacterial agent (Sultana et al., 2010). Many active compounds were produced by plants which contained these active groups (secondary metabolites). Certainly, other chemical components of the extracts could also contribute, although lack of chemical profiling has never been reported on this. It is possible that these compounds are mainly responsible for the antifungal activities observed in this study.

Conclusion

The findings of our study revealed that SBL is a rich source of protein as well as mineral, which could be beneficial to human being, while the extracts strongly inhibited many pathogenic bacterial strains and fungi which can be used for the development of new broad spectrum antibiotics. The SBL leaves are still in use as a traditional herbal medicine, containing a number of useful phytochemicals and providing a scientific data base for further primary health care system.

ACKNOWLEDGMENTS

We (authors) are thankful to Center of Biotechnology and Microbiology (COBAM), University of Peshawar Khyber Pakhtunkhwa (Pakistan) for financial supports.

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African Journal of Pharmacy and Pharmacology Vol. 7(7), pp. 389-396, 22 February, 2013
Available online at http://www.academicjournals.org/AJPP
DOI: 10.5897/AJPP12.1432
ISSN 1996-0816 ©2013 Academic Journals

Full Length Research Paper

Effect of Persian shallot (*Allium hirtifolium* Boiss.) extract on glucokinase (GCK), glycogen phosphorylase and phosphoenolpyruvate carboxykinase (PEPCK) genes expression in diabetic rats

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Accepted 24 December, 2012

There has been a growing interest in hypoglycaemic agents from natural products, especially those derived from plants. In the current survey, hypoglycaemic properties of Persian shallot (*Allium hirtifolium* Boiss) was evaluated by studying mRNA expression levels of the key enzymes involved in carbohydrate metabolism in liver, Glucokinase (GCK), phosphoenolpyruvate carboxykinase (PEPCK), and glycogen phosphorylase. Thirty two male rats were divided into 4 groups of 8, diabetic groups received 100 and 200 mg/kg Persian shallot extract, diabetic control and normal control received 0.9% saline for 30 days. At the end of the experimental period blood and liver samples were collected. FBS and insulin levels were measured and followed by analysis of the gene expression by Real-Time PCR based methods. Findings indicated that the Persian shallot significantly reduces the FBS level in parallel with slight enhancement of insulin in diabetic rats' serum. Investigations of gene expression showed that Persian shallot gently increased GCK and glycogen phosphorylase but decreased PEPCK gene activity, in diabetic rats. In conclusion, the data suggest that Persian shallot is an effective hypoglycaemic agent, the observed effect may be via its ability to enhance insulin secretion and GCK gene expression and to decrease hepatic glucose output by reducing PEPCK.

Key words: Persian shallot, Glucokinase, glycogen phosphorylase, phosphoenolpyruvate carboxykinase, diabetes.

INTRODUCTION

Diabetes mellitus is a common metabolic disorder characterized by hyperglycemia due to defects in insulin production and/or function (Abel et al., 2001). Liver is an insulin-sensitive tissue and plays a major role in maintaining glucose homeostasis via regulating process of the glucose utilization and gluconeogenesis. The liver produces glucose by two pathways, gluconeogenesis (de novo synthesis of glucose) and glycogenolysis (enzymatic break-down of glycogen by glycogen phosphorylase catalytic activity) (Ferre et al., 1996). In the liver, insulin suppresses transcription of genes encoding gluconeogenic

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and glycogenolytic enzymes and stimulates transcription of genes encoding glycolytic enzymes, thus, leading to decreased glucose level (Barthel and Schmoll, 2003). The glucose which is taken up by mammalian cells has to be converted into glucose 6-phosphate by Glucokinase (GCK) as a prelude for further utilization in glycolysis, the pentose phosphate pathway or glycogen synthesis. In the liver, expression of GCK is very closely dependent on the presence of insulin. Hence, GCK mRNA and protein disappear from the livers of insulin deficient rats and is restored following insulin treatment. The central role of GCK in facilitating glucose disposal by the liver on the one side, and insulin secretion by the islets of Langerhans on the other side, provide a strong rationale for the search of small molecule activators of GCK in drug discovery programs aimed at developing a new class of antidiabetic drugs (Iynedjian et al., 1998).

Another important enzyme in carbohydrate metabolism in mammals, including human being is phosphoenol-pyruvate carboxykinase (PEPCK), which is involved in regulation of the circulating glucose level. Gluconeogenic tissues, such as kidney and liver, convert lactate and other non-carbohydrate molecules to glucose, which in turn is released into the circulation (Matte et al., 1997). In mammals, gluconeogenic genes are mainly under insulin control. Insulin itself inhibits expression of PEPCK enzymes at the transcriptional level. The importance of PEPCK in carbohydrate metabolism in humans is that it has the potential to be employed as a potential drug target in the treatment of diabetes mellitus (Barthel and Schmoll, 2003).

Also, increasing number of reports suggested that glycogenolysis inhibitors are probably useful in the treatment of diabetes. Glucose production from the catalysis of glycogen to glucose-1-phosphate is rate-limited by glycogen phosphorylase, a well-studied enzyme that is regulated by multiple covalent, substrate, and allosteric effectors (Newgard et al., 1989).

The underlying goal for all types of diabetes treatment and management is to maintain blood glucose on an adequate level. Considering the heterogeneity of this disease, current therapies are often limited. And to achieve that, the investigation of new compounds with improved antidiabetic action is of paramount importance (Celik et al., 2009). Investigations showed green tea components have glycaemic effects and mimic insulin, and reduces gene expression of the gluconeogenic enzyme PEPCK, and increase Glucokinase mRNA expression in the liver of rats in a dose dependent manner (Nakagawa et al., 2002).

Studies have highlighted the benefits of medicinal plants with combined antidiabetic and antioxidant properties (Nakagawa et al., 2002). Persian shallot (*Allium hirtifolium* Boiss) is a nutritive plant with special taste that belongs to Liliaceae family. It is one of the important edible onions in Iran. It is a native Iranian plant and grows wildly in the Zagross Mountains. Biochemical analysis of Persian shallot extracts has confirmed its hypoglycaemic and hepatoprotective effects (Hosseini et al., 2012. Hosseini-zijoud et al., 2012). There are several reports that emphasized shallot medicinal effects as antioxidant (Leelarungrayub et al., 2006), immune system regulating (Jafarian et al., 2003) and anticancer (Ghodrati et al., 2008). The Persian shallot extract is a stronger hypoglycaemic agent compared to garlic extract and it could be a useful supplemental remedy in diabetes (Leelarungrayub et al., 2006). Since Persian shallot grows as a wild plant only in some mountains of Iran, limited information is available regarding different aspects of this species. Therefore, the present study was designed to investigate the possible hypoglycaemic effects of two different doses (100 and 200 mg/kg) of Persian shallot in streptozotocin-induced diabetic rats; a suitable model for type 1 diabetes (Kopp et al., 2008). We have evaluated glucose homeostasis, the expression of genes regulating glycolysis, gluconeogenesis and glycol-genolysis in liver, as well as insulin and FBS levels.

MATERIAL AND METHODS

Preparation of hydroalcoholic extract of Persian shallot

Fresh Persian shallot (*A. hirtifolium* Boiss) bulbs were obtained from Kangavar (Kermanshah-Iran). The genus and species of the bulbs were confirmed by the botanists (Department of Botany, Valiasr University Rafsanjan-Iran). Then, 100 g of fresh bulbs was well crushed and 400 ml distilled water/ethanol (25/75) was added. After 48 h incubation, the solution was filtered using a filter paper through a Buchner funnel. The filtered resultant solutions obtained from this stage, concentrated by means of a vacuum distillation and decanted to dry powder was used to prepare the needed concentrations (Momeni, 2000).

Induction of diabetes and Persian shallot treatments

In this study 32 male albino Wistar rats weighing 180 to 230 g were recruited. Twenty-four rats were injected (intraperitoneal injection) with 45 mg/Kg body weight of streptozotocin (STZ) (diabetic type-1 rats) and eight rats were included as normal group. After being matched according to body weight, the rats were allocated to four groups of 8:

Group 1: diabetic rats received daily 200 mg/kg Persian shallot extract (2 ml) for 30 days.
Group 2: diabetic rats received daily 100 mg/kg Persian shallot extract (2 ml) for 30 days.
Group 3: diabetic rats received daily 0.9% saline (2 ml) for 30 days (diabetic control).
Group 4: normal rats received daily 0.9% saline (2 ml) for 30 days (normal control).

The solutions (2 ml) given to animals by using a gavage syringe. The animals were then housed in cages and had free access to water and standard food. Animal handling was performed in accordance with the guidelines of Iranian animal ethics society, Rafsanjan University of Medical Science rules and under supervi-

sion of Professor Mehdi Mahmoodi. At the end of 30 days treatment, blood and liver samples were collected and the levels of FBS and insulin were measured in all study groups and gene expression analyzed by Real-Time PCR.

Biochemical analysis

Plasma insulin concentrations were assayed by ELISA method using a commercial kit (Mercodia, Sweden) and FBS was measured by BT-3000 autoanalyzer.

Extraction of RNA

For the isolation of tissue RNA, rats were humanly sacrificed and under aseptic situations the liver tissues were removed and immediately frozen in liquid nitrogen. Prior to RNA extraction, liver samples were homogenized in TRIZOL^{TM} reagent (Invitrogen) using Mixer 301. Total RNA was extracted according to the manufacturer's guidance. RNA samples were electrophoresed in agarose gels and visualized with ethidium bromide for quality control.

cDNA synthesis and quantitative Real-Time PCR

Three micrograms of RNA were reverse transcribed with reverse transcriptase for 1 h at 37°C for synthesis of cDNA. Quantitative changes in mRNA expression were assessed with real-time quantitative Real-Time PCR (Bio-Rad CFX) using SYBR-Green detection consisting of SYBR Green PCR Master Mix (Aria-tous, Iran). The PCR master mix was made up by 0.5U of *Taq polymerase*, 2 µL of each primer and 3 µL of each cDNA samples in a final volume of 20 µL. All amplifications were repeated three times. Oligonucleotide primer sequences are illustrated in Table 1. β_2-microglobulin was used as endogenous control, and each sample was normalized on the basis of its β_2-microglobulin content. Relative quantification of the mRNA expression levels of target genes was calculated using the $2^{-\Delta\Delta Ct}$ method (Celik et al., 2009) (Table 2).

$\Delta\Delta Ct = (C_t \text{ gene studied} - C_t\ \beta_2\text{-microglobulin})_{\text{treated}} - (C_t \text{ gene studied} - C_t\ \beta_2\text{-microglobulin})_{\text{control}}$.

Statistical analysis

Results are presented as mean±SD. Statistical difference between the means of the various groups were analyzed using one way analysis of variance (ANOVA) followed by Tukey's multiple test. Data were considered statistically significant if $p < 0.05$.

RESULTS

FBS and insulin

The FBS concentrations of four groups of rats during experimental period are shown in Figure 1. There was a significant difference in FBS level among all groups and Persian shallot consumption reduced significantly FBS level in diabetic treated groups in dose dependent manner (P<0.05).

The Fasting plasma Insulin levels of four groups of rats during experimental period are displayed in Figure 2. Diabetic groups showed statistically lower insulin levels in compare to normal control. Although Persian shallot consumption slightly increases insulin level in diabetic rats but this elevation was not significant.

The mRNA levels of glycolytic and gluconeogenetic enzymes in liver

The expression level of the genes in the normal control group was considered as 100% and the expression in the other groups were accordingly calculated (Table 2). When compared with control rats, diabetes was found to suppress GCK gene expression in liver (Figure 3). The Persian shallot elevated hepatic glucokinase gene expression when compared with the control group (P<0.05) (Figure 3 and Table 2). In contrast, PEPCK and glycogen phosphorylase genes expression were higher in the diabetic rats (Figures 4 and 5) while, treating with Persian shallot increased glycogen phosphorylase and decreased PEPCK gene expression however the observed effect was not significant (Table 2).

The level of mRNA encoding target genes were detected by Real-Time PCR and were normalizes with B2-microglobulin mRNA (as housekeeping gene) (using the $2^{-\Delta\Delta Ct}$ method). Group 1: diabetic rats received 200 mg/kg Persian shallot. Group 2: diabetic rats received 100 mg/kg Persian shallot. Group3: diabetic rats received 0.9% saline.

DISSCUSION

Chronic insulin deficiency and insulin insensitivity are the major causes of the decreased hepatic glucose utilization and increased glucose production in diabetes, because insulin decreases the hepatic glucose output by activating glycogen synthesis and glycolysis, and by inhibiting gluconeogenesis (You-Gui et al., 2011). Recently there has been a growing interest in hypoglycaemic agents from natural products, especially those derived from plants, because plant sources are usually considered to be less toxic, with fewer side effects than synthetic sources. Several bioflavonoids, ubiquitously present in Persian shallot (*A. hirtifolium Boiss*.) have been reported to improve hyperglycemia in diabetes mellitus (Leelarungrayub et al., 2006). In our study Persian shallot significantly reduced FBS while gently increasing serum insulin level. Recently, several studies have shown that the activities of many enzymes such as GCK, and glycogen phosphorylase in liver of diabetic mice were significantly affected (Palsamy and Subramanian 2009, Zhang et al., 2009). Zhang et al. (2009) reported that glucokinase enzyme activity was decreased by more than

Table 1. Primers sequences.

Transcripts	Primer sequences
Glucokinase (GCK)	F-5'ACTGACTATCCGGCTACATG3' R-5'GATTCCTGCTTGAATAGTGC3'
phosphoenolpyruvate carboxykinase (PEPCK)	F-5'GTCACCATCACTTCCTGGAAGA3' R-5'GGTGCAGAATCGCGAGTTG3'
Glycogen phosphorylase	F-5'CCCGAGCACCCAATGACTTTAACC3' R-5'GCGAGTGCGGGATGTGTGTCA3'
β2-microglobulin	F-5'TTCTGGTGCTTGTCTCACTGA3' R-5'CAGTATGTTCGGCTTCCCATTC3'

Table 2. Real-Time PCR results for selected genes.

Groups / Genes	Group 1 (Real-Time PCR fold changes)	Group 2 (Real-Time PCR fold changes)	Group 3 (Real-Time PCR fold changes)
Glucokinase (GCK)	1.46	1.28	1.o1
Phosphoenolpyruvate carboxykinase (PEPCK)	1.22	1.47	1.89
Glycogen phosphorylase	0.67	0.56	0.51

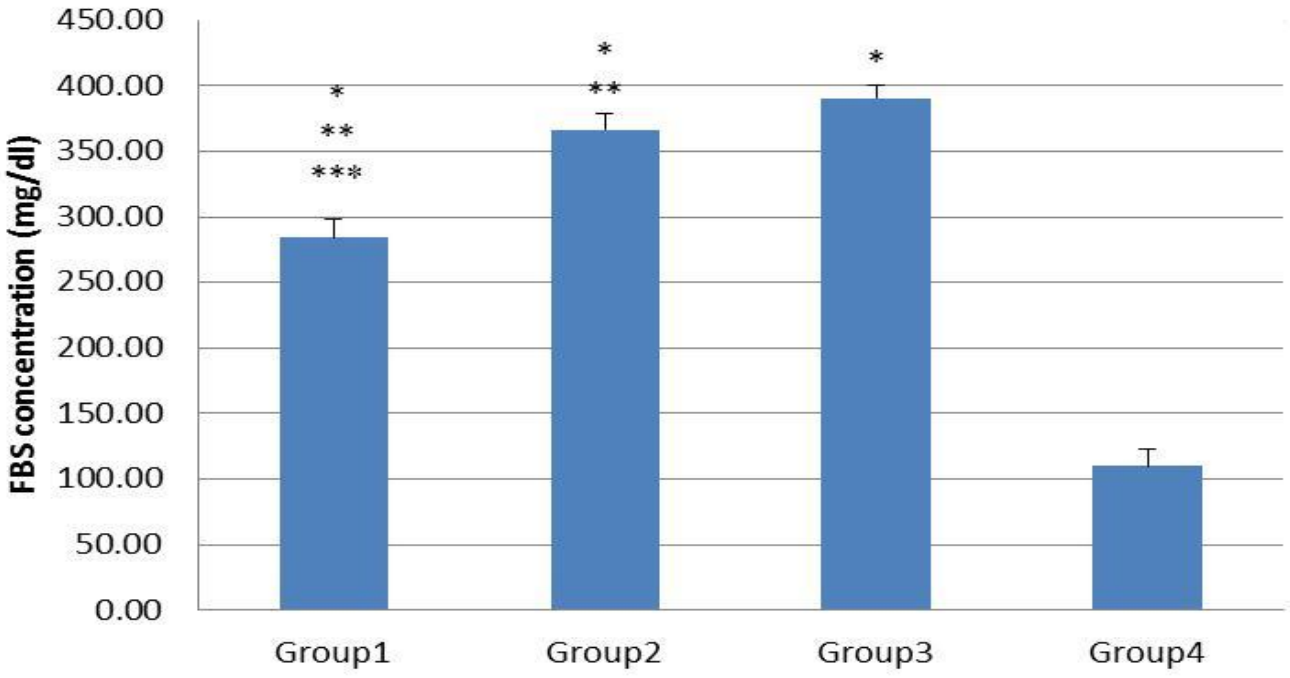

Figure 1. The effect of different concentration of Persian shallot on FBS level (mg/dl). (Mean±SD) (P<0.05).
Group1: diabetic rats received 200 mg/kg Persian shallot. Group2: diabetic rats received 100 mg/kg Persian shallot. Group3: diabetic rats received 0.9% saline. Group4: normal rats received 0.9% saline. * Significant differences with Group 4 (P<0.05). ** Significant differences with Group 3 (P<0.05). *** Significant differences with Group 2 (P<0.05).

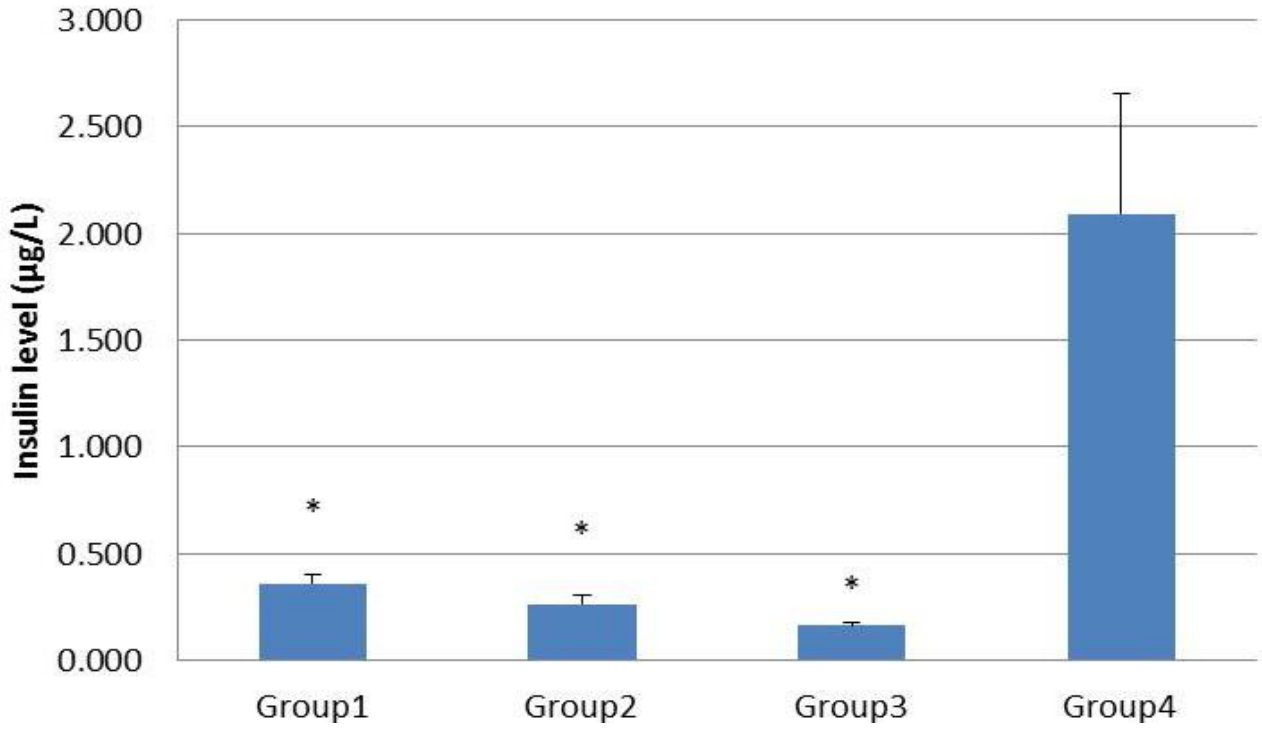

Figure 2. The effect of different concentration of Persian shallot on Insulin level (µg/L). (Mean±SD) (P<0.05).
Group1: diabetic rats received 200 mg/kg Persian shallot. Group2: diabetic rats received 100 mg/kg Persian shallot. Group3: diabetic rats received 0.9% saline. Group4: normal rats received 0.9% saline. * Significant differences with Group 4(P<0.05).

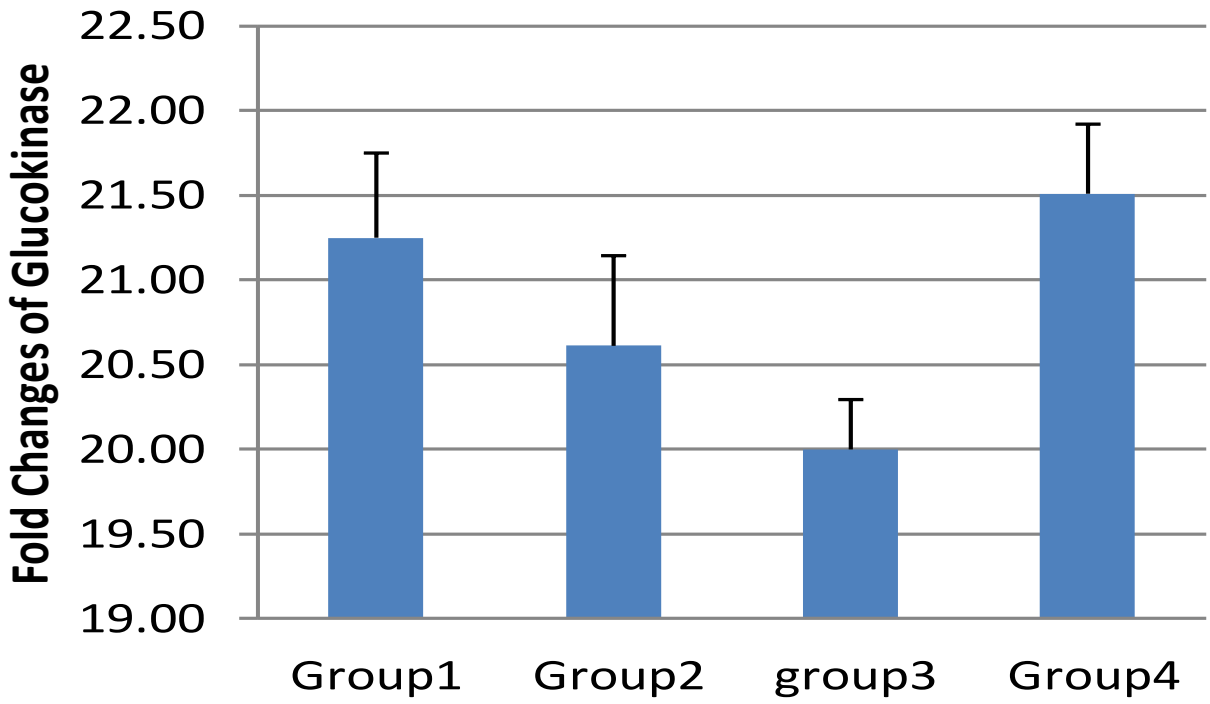

Figure 3. The expressed levels of Glucokinase mRNA (fold) in all groups (using ANOVA test, Mean±SD) (P< 0.05).
Group1: diabetic rats received 200 mg/kg Persian shallot. Group2: diabetic rats received 100 mg/kg Persian shallot. Group3: diabetic rats received 0.9% saline. Group4: normal rats received 0.9% saline.

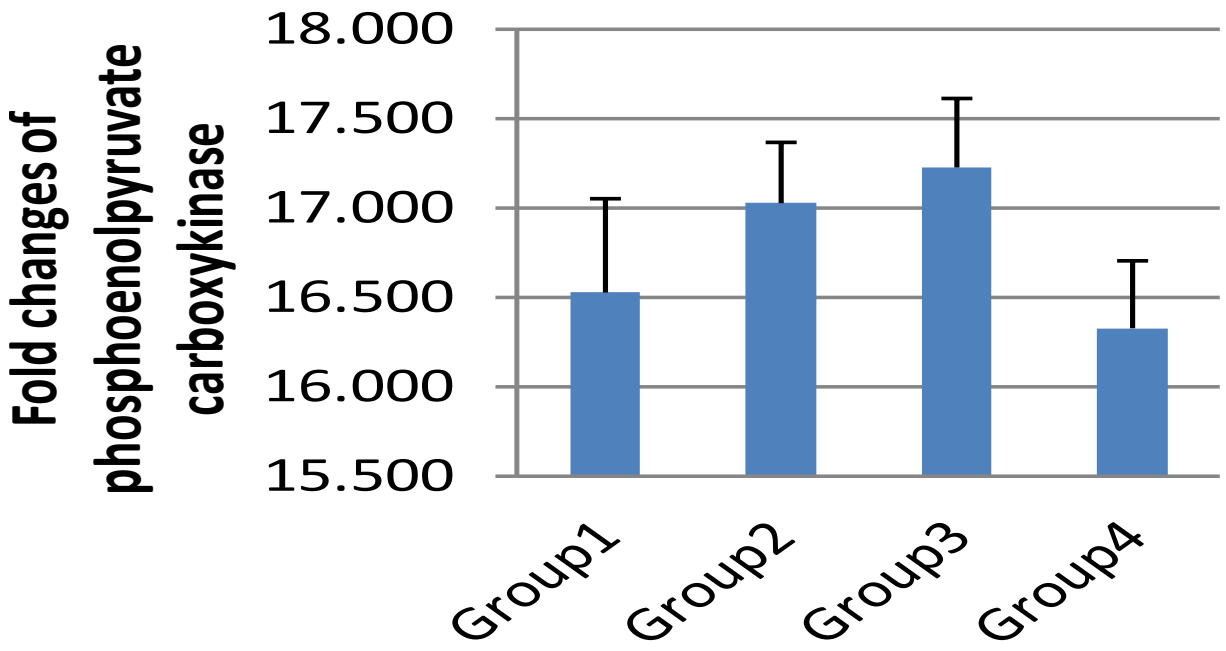

Figure 4. The expressed levels of phosphoenolpyruvate carboxykinase mRNA (fold) in all groups. (using ANOVA test, Mean±SD) (P< 0.05).
Group1: diabetic rats received 200 mg/kg Persian shallot. Group2: diabetic rats received 100 mg/kg Persian shallot. Group3: diabetic rats received 0.9% saline. Group4: normal rats received 0.9% saline.

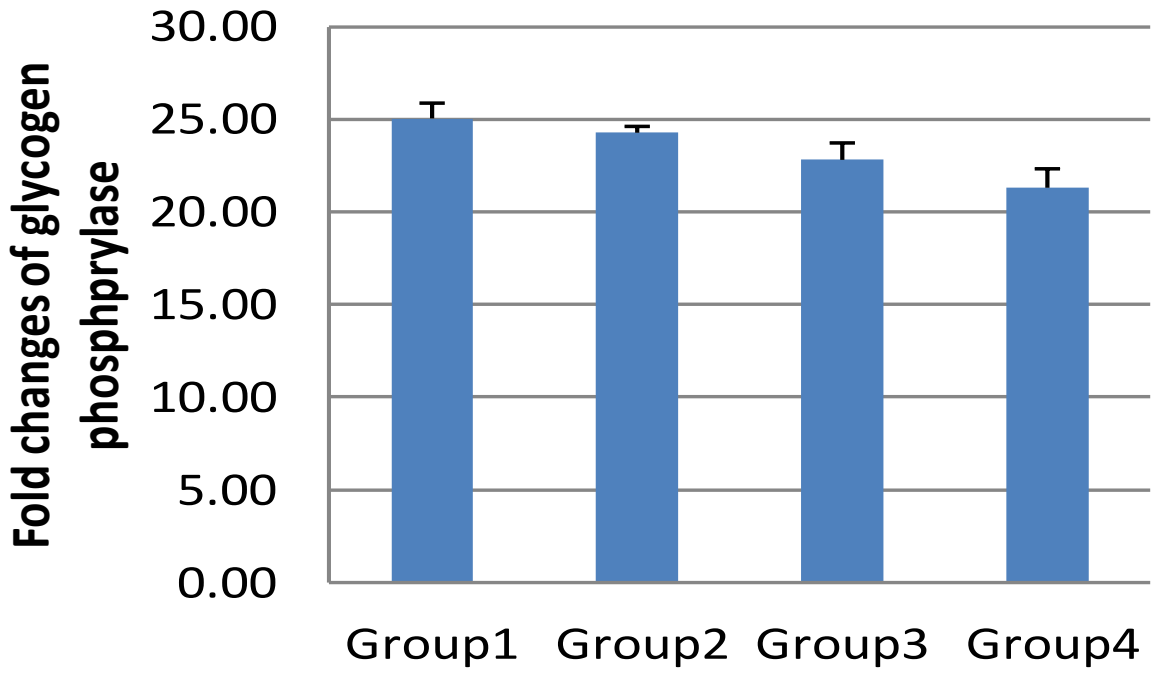

Figure 5. The expressed levels of glycogen phosphprylase mRNA (fold) in all groups (using ANOVA test, Mean±SD) (P<0.05).
Group1: diabetic rats received 200 mg/kg Persian shallot. Group2: diabetic rats received 100 mg/kg Persian shallot. Group3: diabetic rats received 0.9% saline. Group4: normal rats received 0.9% saline.

90% in the liver of diabetic rats. So in this study, to evaluate the antidiabetic mechanism(s) of Persian shallot, the key enzymes of carbohydrate metabolism such as GCK, PEPCK, and glycogen phosphorylase were investigated in liver at mRNA level using Real-Time PCR. We showed that hepatic GCK was down regulated, but hepatic PEPCK and glycogen phosphorylase were up-regulated evidently in diabetic rats. It also increased insulin level in serum, these results suggest that the antioxidants could restore the damaged pancreas and stimulate the secretion of pancreatic insulin at the same time. The Persian shallot has probable ability to accelerate the hepatic glucose metabolism may be via regulating the expression of the functional genes of PEPCK, and GCK. The results of Real-Time PCR studies provided supportive evidence for FBS analyses (Jung et al., 2004). In fact, this hypoglycaemic action of Persian shallot is likely to be associated with a marked enhancement of the GCK mRNA expression in the liver. Current results is consistent with previous studies that showed GCK mRNA expression increase in Naringin (Jung et al., 2004) and epigallocatechin gallate, a main polyphenolic constituent of green tea (Waltner et al., 2002) treated rats. Hepatic GCK has a major effect on glucose homeostasis and is a potential target for pharmacological treatment of diabetes, and rats overexpressing GCK in the liver had reduced blood glucose. The elevation of hepatic GCK can cause an increased utilization of the blood glucose for energy production or glycogen storage in the liver (Jung et al., 2006). A low hepatic GCK activity is also reported to favor the release of glucose synthesized by gluconeogenesis into the circulation. The PEPCK is a key enzyme that controls gluconeogenesis and glucose output from the liver. It is involved in the synthesis of glucose-6-phosphate from non-carbohydrate precursors. Enhanced expressions of the PEPCK gene in liver was present in most models of diabetes, and is thought to contribute to the increased hepatic glucose output seen in this disease. Insulin is the most important hormone that inhibits gluconeogenesis. At the gene transcription level, insulin down-regulated the mRNAs encoding PEPCK (Davies et al., 2001). In the present study PEPCK gene expressions was increased in diabetic rats whereas reduced in Persian shallot treatment group. The present finding is in agreement with Jung et al. (2006) observation that Caffeic acid phenethyl ester markedly reduces PEPCK mRNA levels in diabetic rats. In addition, previous investigations showed that Naringin (Jung et al., 2004) and epigallocatechin gallate, a main polyphenolic constituent of green tea (Waltner et al., 2002) suppress PEPCK mRNA expression. This is consistent with present findings. In addition to PEPCK, GCK activity is also reported to be controlled primarily at the level of transcription, being regulated by insulin. High insulin levels have been shown to inhibit hepatic glucose production by means of stimulation of GCK gene transcription (Friedman et al., 1997). In fact in the present study, the changes in hepatic glucose-regulating enzymes could be partly attributed to insulin levels because plasma insulin level was elevated in Persian shallot-supplemented diabetic rats in comparison to the control (Jung et al., 2006). Further, previous investigations indicated that hepatic glycogenolysis plays a major role in the regulation of plasma glucose levels in diabetic rats which suggest that glycogen phosphorylase inhibitors may be useful in the treatment of diabetes (Martin et al., 1998). Surprisingly Persian shallot increases glycogen phosphorylase in diabetic rats. It has been demonstrated that insulin inhibits glycogenolysis via stimulation of glycogen phosphorylase activity (Celik et al., 2009), but in the current study Persian shallot increased insulin and glycogen phosphorylase activity simultaneously.

Conclusions

Overall, based on the finding of the present study it could be suggested that Persian shallot has complimentary potency to develop as a hypoglycaemic agent for treatment of diabetes mellitus. Moreover, Persian shallot perhaps also modulates the hepatic glucose metabolism by balancing (up/down-regulating) the expression of rate-limiting enzymes (GCK, PEPCK and glycogen phosphorylase) in STZ-induced diabetic mice. The antioxidant activity of Persian shallot is possibly capable of protecting pancreatic islets from STZ-induced damage by scavenging the free radicals and repairing the destroyed pancreatic β-cells, and in fact guarantees the normal secretion of insulin in serum.

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African Journal of Pharmacy and Pharmacology Vol. 7(7), pp. 397-404, 22 February, 2013
Available online at http://www.academicjournals.org/AJPP
DOI: 10.5897/AJPP12.1470
ISSN 1996-0816 ©2013 Academic Journals

Full Length Research Paper

Detection, biological effectiveness, and characterization of nanosilver-epidermal growth factor sustained-release carrier

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Accepted 29 October, 2012

A nanosilver-epidermal growth factor sustained-release carrier was synthesized with the self-assembly method and characterized by transmission electron microscopy and Ultra violet (UV) spectrophotometry. The biological activity of the sustained release carrier was determined through cytological, bacteriological, and wound-healing experiments. The results show that the nanosilver-epidermal growth factor sustained-release carrier was well dispersed, with uniform particle size and that it had good antibacterial properties that were similar to those of nanosilver. Nanosilver-epidermal growth factor sustained-release carrier is superior to epidermal growth factors in effectively promoting cell division and proliferation. The results of wound-healing experiments show a curative effect.

Key words: Epidermal growth factor, nanosilver, antibacterial, wound, cell proliferation.

INTRODUCTION

Nanoparticles have been increasingly applied in biomedical, pharmaceutical, and clinical medicine. Among them, nanosilver is widely used in clinical burn, dental, and urologic practice (Kim et al., 2007; Chekman et al., 2011; Dallas et al., 2011). In cytotoxic and animal experiments, Arorat et al. (2008), Ai et al. (2011) and Teow et al. (2011) proved that nanosilver has no toxicity, but has high antibacterial activity. Tian et al. (2007) found that local use of nanosilver wound dressing could not only accelerate healing, but also improve the appearance of scars. Madhumathi et al. (2010) and Ong et al. (2008) showed that nanosilver/chitosan dressing effectively resists *Staphylococcus*, colonic, and other bacteria and shows good hemostatic effects in treatment of burn wounds. Growth factors are a class of peptides or proteins that can regulate cell growth and differentiation and promote tissue healing (Xie et al., 2006). Epidermal growth factor (EGF) achieved good clinical results (Kiyohara et al. 1991) but its *in vivo* stability is poor; it is vulnerable to degeneration or inactivation and is easily diminished from blood circulation. Development of a stable, safe, and effective preparation has become a challenging and practical focus in pharmacy research (Değim et al., 2011).

Existing studies have demonstrated the role of controlled- or sustained-release formulations that are prepared with nanoparticles as a carrier. They can effectively protect drugs against inactivation and achieve sustained or controlled release, even targeted release, thus significantly improving the curative efficacy and reducing toxic side effects (Vaiana et al., 2011).

Anti-inflammatory nanosilver combines with EGF to create a sustained-release carrier that has both resistance to infection and sustained-release properties similar to those of EGF. The resultant carrier can promote skin damage repair and make up for the wound infection-induced low activity when EGF is used alone. Our research group has preliminarily determined the optimal particle size and complex conditions for nanosilver and EGF. In this study, we prepared a sustained-release carrier using a 20-n nanosilver particle and EGF by the self-assembly method (Janjua et al., 2011) to elucidate the characterization and biological effects of the carrier.

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MATERIALS AND METHODS

Preparation of silver nanoparticle-EGF sustained-release carrier solution

A 5,000 ppm solution of silver nanoparticles was magnetically stirred with 50 µg/mL EGF solution (10 mL) in a 5 mL sterile flask, and the solution was adjusted with HCl-Tris buffer to pH 7.0. The volume was set at 50 mL and scattered for 30 min with an ultrasonic dispersion machine. The solution was placed in a 37°C water bath overnight for 12 h to obtain a 500 ppm solution of sustained-release carrier (final concentration of silver nanoparticles, 500 ppm; final concentration of EGF, 10 µg/mL). The sustained-release solution (25 ppm) was similarly prepared (final concentration of silver nanoparticles, 25 ppm; final concentration of EGF, 10 µg/mL). Freeze-dried EGF powder (1 mg) was dissolved in sterile distilled water to prepare the EGF-alone group (10 µg/mL) and nanosilver-alone group (100 ppm). All solutions were stored in a sterile bottle in a refrigerator at 4°C for further use.

Characterization of silver nanoparticle-EGF sustained-release carrier solution

Transmission electron microscopy

The 500 ppm nanosilver particle solution and 500 ppm silver nanoparticle-EGF sustained-release carrier solution were observed with transmission electron microscopy.

Ultraviolet visible spectrophotometry

The 25 ppm sustained-release carrier solution was centrifuged at 20,000 rpm for 2 h and the supernatant was then collected for detection. Double distilled water served as a reference sample adjusted to “A0.000.” The test sample was placed into the cuvette followed by the ordered measurement of the EGF-alone group (10 µg/mL), nanosilver-alone group (100 ppm), nanosilver-EGF sustained-release carrier group (25 ppm), and sustained-release supernatant group.

Cell proliferation experiments with nanosilver-EGF sustained release carrier

Skin fibroblast cell line KMST6 was resuscitated and added to culture medium to obtain a triturated cell suspension. The cell suspension at 5×10^5/mL was subpackaged and cultured in a 75 mL culture flask at 37°C in a 5% CO_2 incubator until passages of 4 to 8. The suspension was then seeded onto a 96-well cell culture plate with 100 µL in each hole at 37°C in saturated humidity of 5% CO_2 for 24 h. The culture medium was discarded and the sample was added to the EGF (10 mg/L) group, nanosilver solution (500 ppm) group, nanosilver-EGF (500 ppm) group, nanosilver-EGF combination group, and control group. Each group was set at eight double holes; there were four repeated plates in each group with 100 µL of solution in each hole. All samples were cultured at 37°C in a 5% CO_2 incubator, and one culture plate was taken out at 12, 24, 36, and 48 h for an MTT colorimetric test. The cell growth curve was plotted.

Antibacterial test with nanosilver-EGF sustained-release carrier

Five pathogenic microorganisms, namely *Staphylococcus aureus* (ATCC 29213), *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (10102), *Candida albicans*, and *Streptococcus pneumoniae* were incubated with the culture medium (including nutrient broth medium and agarose medium) at 4°C. Each bacterial species was repeatedly incubated on five plates; the concentration of bacterial suspension was estimated turbidimetrically and inoculated onto petri dishes at concentrations of 5×10^5 to 5×10^6 cfu/mL. The bacterial suspension was smeared onto the surface of a nutrient agar plate, and the petri dishes were dried at room temperature. The sterile, dried filter paper (5 mm diameter) was collected and added to 5 µL of reagents to prepare antibacterial slices. Samples were cultured for 24 h in a 37°C incubator. The diameter of the antibacterial ring was measured with compasses and a caliper; measurements were repeated three times.

Wound healing experiments with nanosilver-EGF sustained-release carrier

One wound was made on each side of the spinal cord in 15 rats which were intramuscularly injected with gentamicin at 5 mg/kg (equivalent to the plasma concentration in adults) once daily for 3 days for systemic anti-infective treatment. All wounds were randomly assigned to the nanosilver-EGF sustained-release carrier group (NanoAg-EGF), nanosilver group (NanoAg), EGF-alone group, nanosilver-EGF combination group (NanoAg+EGF), or saline control group. The two wounds were treated by different means once daily. The drugs infiltrated the whole wound by sterile syringe infusion, and each wound received 0.25 mL of drug per treatment. Wounds were photographed on days 3, 7, and 12 and at the time of wound healing, and the non-healing area was calculated using a computer image analysis system (CAD software). The healing rate was calculated as follows: Healing rate = (initial wound area – nonhealing area) / initial wound area × 100%.

Statistical analysis

Measurement data are expressed as mean ± standard deviation and were analyzed using SPSS 13.0 software. Differences among groups were compared using analysis of variance. Pairwise comparison was performed with the LSD test.

RESULTS AND DISCUSSION

Characterization of nanosilver-EGF sustained-release carrier with transmission electron microscopy

A transmission electron microscopic image of nanosilver at 500 ppm is shown in Figure 1. The silver nanoparticles were spherical with uniform distribution, showing no agglomeration or growth, and the particle size was about 15 to 25 nm. A transmission electron microscopic image of the nanosilver-EGF sustained release carrier is shown in Figure 2. Lightly stained EGF covered the surface of the spherical silver nanoparticles and formed a nebula-like shadow, which was surrounded by silver nanoparticles.

Peptides and proteins are increasingly used in clinical practice, and the preparation method of nanoparticles has been developed (Zhao et al., 2008; Zheng et al., 2006) so that they may serve as carriers of peptide and protein drugs. The use of biodegradable polymers or inorganic nanoparticles as carriers of peptides and proteins, thus achieving a sustained-release effect, is a

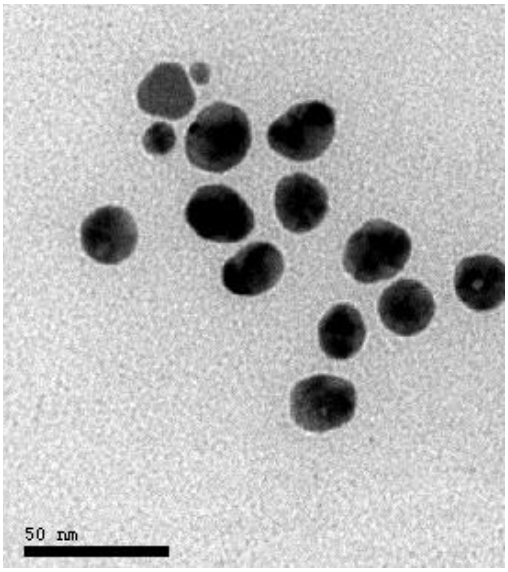

Figure 1. Transmission electron microscopic photos of silver nanoparticles (×500,000).

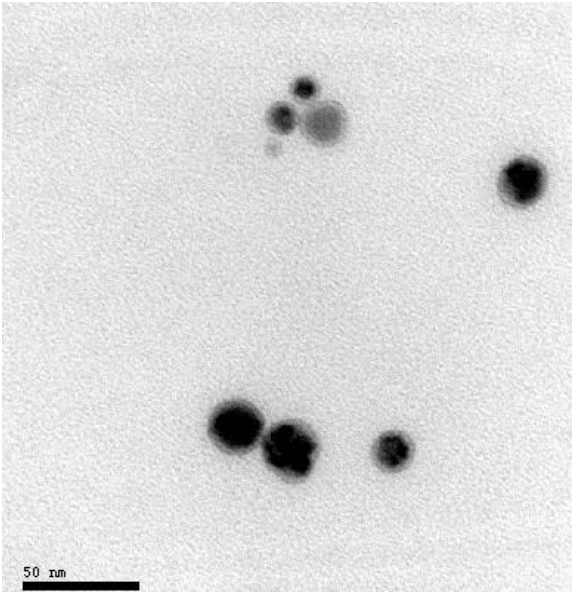

Figure 2. Transmission electron microscopic photos of nanosilver-EGF complex (×500,000).

current research focus (Tan et al., 2010; Ana et al., 2011; Harde et al., 2011).

In this study, transmission electron microscopy characterization analysis showed that the obtained silver nanoparticles were evenly distributed and close to spherical in shape, and there was no agglomeration or growth. Transmission electron microscopic images of the nanosilver-EGF sustained-release carrier displayed that the carrier was well dispersed in the solution with no reunion or growth and that the lightly dyed EGF adhered to the surface of the spherical silver nanoparticles, forming a nebula-like shadow surrounded by silver nanoparticles. This is objective evidence of EGF adhesion on the silver nanoparticles.

UV-VIS characterization

The UV visible absorption spectra of nanosilver solution, EGF solution, and nanosilver-EGF sustained release solution are shown in Figure 3. The first absorption peak in curve 4 was exactly the same as that in curve 1, indicating that free EGF was present in the nanosilver-EGF sustained-release solution. The second absorption peak of curve 4 shifted to the right compared with the absorption peak in curves 2 and 3, with a 5 nm gap; that is to say, the UV visible absorption peak of the nanosilver-EGF sustained-release carrier was 5 nm away from that of the silver nanoparticles. This evidence suggests that the EGF acting with nanosilver in the nanosilver-EGF sustained-release solution produced a nanosilver-EGF complex. According to the MieTheory (Papoff et al., 2011; Bhandari et al., 2011), the plasma absorption peak gradually red-shifts with increasing nanoparticle size. When the size of silver nanoparticles increased, the plasma absorption peak red-shifted, which is strong evidence for nanosilver adhesion on EGF. EGF effectively adsorbed to the surface of silver nanoparticles, indicating that the nanosilver-EGF sustained-release carrier was successfully prepared. This is consistent with a previously described outcome (Tsai et al., 2011) showing that silver-nanometer particles can act with proteins, resulting in the alteration of its spectrum.

Nanosilver-EGF sustained-release carrier promoted cell proliferation

Growth of fibroblast cell culture

Under light microscopy, the number of fibroblasts was increased, the distribution was dense in the whole field of vision, and the cell shape was mostly spindle. Hematoxylin-eosin staining showed pink-stained cytoplasm and blue-stained nuclei. Cells were fusiform-shaped with several processes or star-shaped and flat. Their outlines were clear and their nuclei were oval. There were no significant differences in the morphology of the treated cells (Figure 4).

Nanosilver-EGF sustained-release carrier promoted cell proliferation

The absorbance value in each group was detected at 12, 24, 36, and 48 h (Figure 5). There was no significant difference in the absorbance value of human fibroblasts at 12 h (0.180 ± 0.011 versus 0.186 ± 0.009; $P > 0.05$).

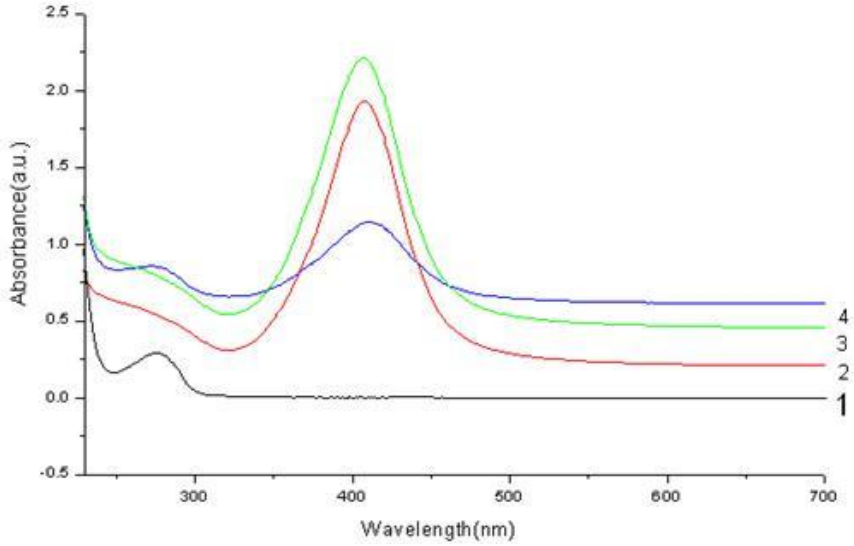

Figure 3. UV absorption spectrum of nanosilver-EGF complex. (1) Absorption spectrum of complex supernatant, (2, 3) absorption spectrum of nanosilver-alone group (100 and 500 ppm, respectively), (4) absorption spectrum of nanosilver-EGF sustained-release group (25 ppm).

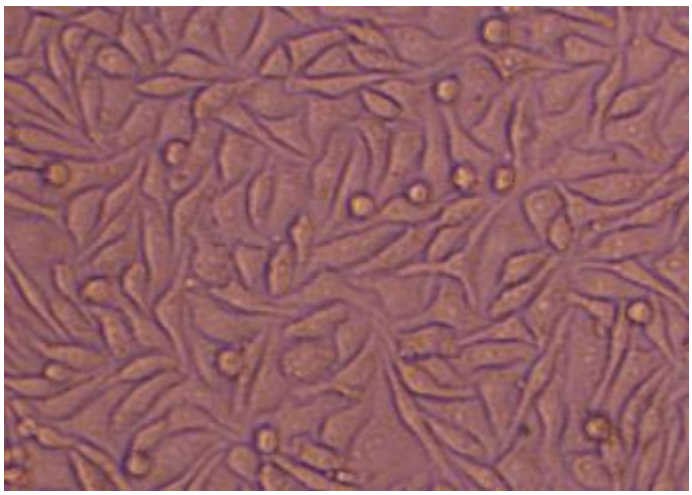

Figure 4. Light microscopic photo of human dermal fibroblasts at 36 h after hematoxylin-eosin staining in the nanosilver-EGF complex group.

Cell proliferation was apparent as time went by. Cell proliferation in groups containing EGF (that is, NanoAg-EGF, NanoAg+EGF, and EGF groups) was significantly apparent, compared with that in the nanosilver and control groups ($P < 0.05$). At 36 and 48 h, cell proliferation in the sustained-release carrier group was the most obvious. Absorbance values were 0.359 ± 0.027 and 0.467 ± 0.026, respectively, which were significantly greater than those in the combined group (0.324 ± 0.022 and 0.359 ± 0.027) and EGF group (0.316 ± 0.019 and 0.357 ± 0.016; $P < 0.05$). This is evidence that cell proliferation was faster and more stable after 24 h in the sustained-release carrier group. Accordingly, we speculate that the nanosilver-EGF sustained-release carrier can greatly promote cell proliferation and that this ability is closely attributed to the sustained-release effect of silver nanoparticles on EGF.

In the cell proliferation experiments, cell proliferation was promoted to varying degrees in the different groups, but no significant statistical difference was found. The reason may be because the biological effects on promotion of cell proliferation remained in the initial phase within a short duration of EGF action. Cell protein and DNA and RNA synthesis was significantly increased,

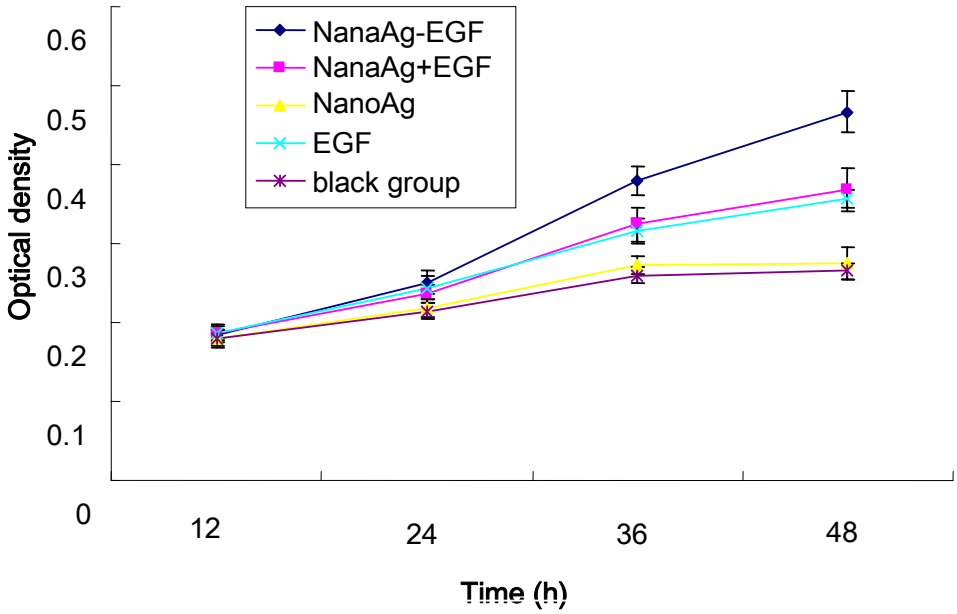

Figure 5. Light microscopic photo of human dermal fibroblasts at 36 hours after hematoxylin-eosin staining in the nanosilver-EGF complex group.

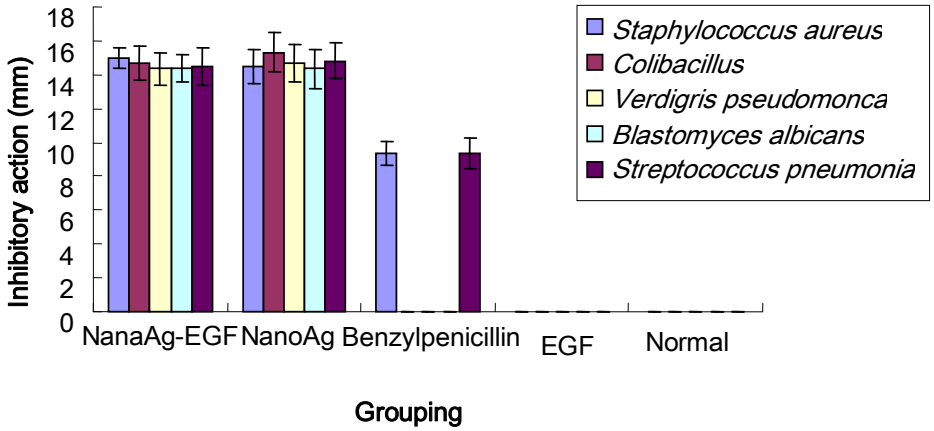

Figure 6. Experimental group's inhibitory action on five pathogenic microorganisms. (1) NanoAg-EGF group, (2) NanoAg group, (3) Benzylpenicillin group, (4) EGF group, (5) Normal control group.

but no quantitative change in cell number was found. When fibroblasts are treated with EGF, the cell cycle duration is about 10 h and DNA synthesis starts at 8 h and becomes active. After 24 h of cell culture, the number of cells increased in the NanoAg-EGF, EGF, and NanoAg+EGF groups with significant differences, compared with the nanosilver and control groups. This is evidence that EGF promoted cell proliferation. After 36 h of cell culture, the number of cells in the NanoAg-EGF group was significantly higher than that in the EGF and NanoAg+EGF groups, suggesting that the concentration of sustained-release carrier was better than that of EGF and explaining the cell proliferation at 36 and 48 h.

Antibacterial test of nanosilver-EGF sustained-release carrier

The inhibitory action on five pathogenic microorganisms in each treatment group was compared. As shown in Figure 6, the NanoAg-EGF and NanoAg groups showed good antibacterial properties on five pathogenic microorganisms, and there was no statistically significant difference in antimicrobial resistance to the five pathogenic microorganisms between the two groups ($P > 0.05$). In the positive-control benzylpenicillin group, antibacterial activity against only *S. aureus* and *Str. pneumoniae* was weak and was significantly lower than

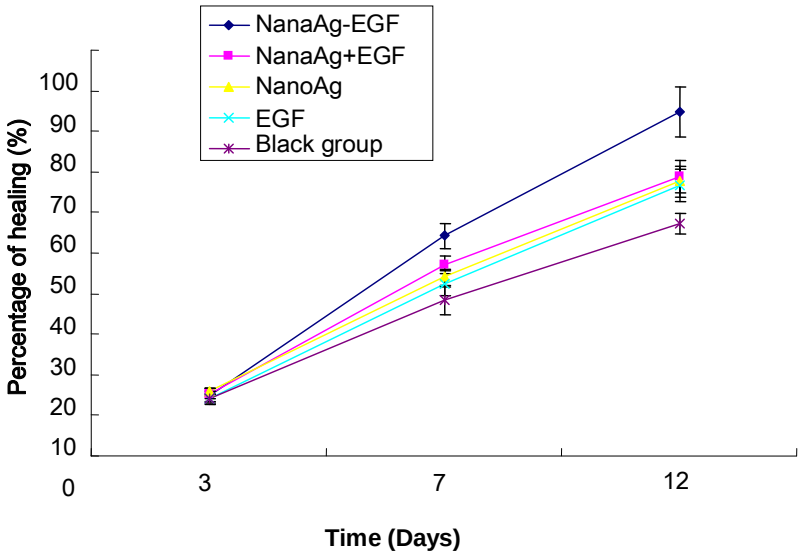

Figure 7. Comparison of wound-healing rate in each group at post-traumatic 3, 7, and 12 days.

that in the NanoAg-EGF and NanoAg groups ($P < 0.05$). In the normal saline and EGF groups, there was no antibacterial effect on the five pathogenic microorganisms.

The antibacterial effect of the nanosilver-EGF sustained-release carrier was determined. Both the NanoAg-EGF and nanosilver groups showed strong inhibitory actions on the five pathogenic organisms, namely *S. aureus*, *E. coli*, *P. aeruginosa*, *C. albicans*, and *Str. pneumoniae*, while the EGF-alone and saline control groups showed no inhibitory effect. In the positive-control group, benzylpenicillin sodium was only resistant to *S. aureus* and *Klebsiella pneumoniae*, and the antibacterial effect was significantly lower than that in the nanosilver and NanoAg-EGF groups. There was no significant difference between the nanosilver and NanoAg-EGF groups. The present experiments not only validate the antibacterial effect of nanosilver, but also confirm that nanosilver has a good inhibitory effect on *S. aureus* and *P. aeruginosa*, which readily demonstrate drug resistance.

Nanosilver-EGF sustained-release carrier promoted wound healing

Morphological observation

The wounds in rats of the NanoAg-EGF group were cleaner than those in the other groups, with less leakage, a mental status that was close to that of normal rats, a normal diet, vigorous activity, and no hair removal. Wound healing in the other groups was relatively poor or even difficult, with more secretions and surrounding swelling, leading to formation of chronic ulcers.

Nanosilver-EGF sustained-release carrier promoted wound healing in animal experiments

The statistical data of the wound-healing rate in each group are shown in Figure 7. The wound-healing rate at 3 days after treatment in each group ranged from 14.105 ± 1.098% to 15.814 ± 1.518%, with no significant difference between groups ($P > 0.05$) at 7 and 12 days. The healing rates in the NanoAg-EGF group were 54.19 ± 3.1137% and 84.933 ± 6.147%, respectively, which were significantly higher than those in the other four groups ($P < 0.05$). The wound-healing duration in the NanoAg-EGF group was 14.75 ± 1.603 days, which was significantly shorter than that of the other four groups (combination group, 17.25 ± 1.422 days; EGF group, 20.167 ± 1.697 days; nanosilver group, 17.083 ± 1.505 days; and control group, 20.333 ± 1.303 days; $P < 0.05$). Thus, the wound-healing rate and duration were the highest and shortest, respectively, in the NanoAg-EGF group.

Many measures are used to improve the wound-healing duration and quality, such as infection control, active removal of necrotic tissue, correction of metabolism, and application of exogenous growth factors. Wound healing is a key issue in plastic surgery and related research, and how to speed wound healing is an expected issue in clinical research (Zhou et al., 2004). In this study, there was no significant difference in the wound-healing rate of each group at 3 days after surgery, indicating that EGF was ineffective in the promotion of wound healing in the early inflammatory stage. Even if anti-infection measures are performed in a timely manner, wound edema and acute infection occur in the post-traumatic 2 to 3 days. Because of the dramatic change in the surrounding environment, cells on the wound surface are still in the shock stage and growth

factors are not available. In addition, a certain amount of time is required to upregulate the exogenous EGF receptor; thus, no promotion of wound healing was found in the NanoAg-EGF group at 3 days.

The healing rate reached a peak in the NanoAg-EGF group 7 days after the injury, with significant differences compared with the other groups; the differences were most significant with time. The wound-healing duration in the NanoAg-EGF group was 4 to 5 days shorter than that in the saline group and 2 to 3 days shorter than that in the combination group. Although the combination group showed a better ability to promote wound healing at all time points, there was no significant difference compared with the EGF-alone and nanosilver groups. Therefore, we speculate that the combination of silver nanoparticles with EGF cannot lead to a qualitative change in wound healing.

In the NanoAg-EGF group, the healing rate was significantly higher than that in the other groups at 7 days, suggesting that our new formulations can avoid wound hydrolysis, induce a sustained and steady release of EGF, and protect factors from wound hydrolysis and bacterial destruction before the adherent growth factor detaches from silver nanoparticles. When the amount of growth factors on the wound surface decreases, the adherent growth factor gradually becomes free from the nanosilver and then binds with receptors that can repair cells and promote cell proliferation. Therefore, the wounds maintain a relatively high concentration of growth factors, and wound healing is accelerated.

Conclusion

All our experimental findings confirm that the herein described nanosilver-EGF sustained-release solution can disperse well, that EGF adheres to the surface of silver nanoparticles, and that growth factor activity and antimicrobial resistance coexist and can effectively promote wound healing. Further studies are required to conclusively determine the clinical application and significance of these results.

ACKNOWLEDGENMENT

This research was supported by The Fundamental Research Funds for the Central Universities (No.2011JQ028), Hunan Provincial Science and Technology Plan Projects (2008SK3114; 2010SK3113), Science and Research Funds of Hunan Health Department (B2007086).

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