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Full Length Research Paper

Anti-inflammatory and analgesic activities of extracts from *Balanites aegyptiaca* L. Delile (Balanitaceae) root bark: Plant used against liver diseases in Bukina Faso

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***Balanites aegyptiaca* (L.) Del** has been used in various traditional medicines against inflammation and pain. The aim of this study was to evaluate the enzymatic, anti-oedematous and analgesic activities of ethanolic and aqueous extracts of *Balanites aegyptiaca* (L.) Del root bark in mice. The extracts were tested *in vitro* for their ability to inhibit the enzymes cyclooxygenase (COX)-1 and COX-2, 15-lipoxygenase (LOX) and phospholipase (sPLA₂). The anti-inflammatory and analgesic activity of ethanolic and aqueous extracts was determined by oral administration to healthy animals at doses of 100, 200, 400 and 600 mg/kg and their involvement in K_{ATP} pathways was verified. The percentage inhibition of the activity of the enzyme phospholipase A₂ was close to 50%, those of COXs (COX 1 and COX 2) comparable to those of the control. It has an ability to inhibit 15-LOX even if the IC₅₀ is lower than that of the reference compound (Zileuton). Pretreatment with the extract at doses of 100 to 600 mg/kg significantly reduced carrageenan-induced edema from 54.91 to 71.80%. The action of the extract significantly decreased the number of contortions with a percentage inhibition greater than 50% for doses of 400 and 600 mg/kg. The action of the extract is not involved in potassium channels (K_{ATP}) in nociception. Pharmacological observed activities provide the scientific basis for the medicinal use of the plant in the treatment of acute inflammation.

Key words: *Balanites argyptiaca*, phospholipase A₂, cyclooxygenases, lipoxygenase, anti-edema activity, analgesic.

INTRODUCTION

Hepatitis is defined by inflammation of the hepatic parenchyma, associated with a more or less extensive necrosis of hepatocytes (Tata Kadiatou et al., 2018). This inflammation is the response to multiple aggressions of liver (virus, drug, toxins or autoimmune, etc), epithelial cells, endothelial cells and infiltrating inflammatory cells (Juhn et al., 2008). Infiltrating inflammatory cells produce the mediators of inflammation which are proteins, peptides, glycoproteins, cytokines, metabolites of arachidonic acid (prostaglandins and leukotrienes), nitric oxide and oxygen free radicals with the potential to fight infection, but also to damage the host (Juhn et al., 2008). Free fatty acids, such as arachidonic acid, substrate for cyclooxygenase (COX 1 and 2) and lipoxygenase (LOX), are products of the hydrolysis of phospholipids by phospholipase A₂ enzymes. These are involved in many cellular mechanisms, including membrane lipid digestion and uptake, angiogenesis, cell proliferation and migration, and innate immunity and in inflammatory diseases (Murakami et al., 2011). Pain and inflammation occur as nonspecific indicator of numerous disease conditions (Abiye et al., 2019). According to Belemilga et al. (2019), pain is well defined as an unpleasant sensory and emotional experience that is associated with possible or actual tissue damage. Inflammations are accompanied most often by pain that can be acute or chronic. Steroidal and nonsteroidal anti-inflammatory drugs are generally used to treat the inflammatory process and block the action of certain pro-inflammatory enzymes. Currently, non-steroidal anti-inflammatory drugs (NSAIDs) are widely prescribed because of their efficacy in the management of pain, inflammation, and rheumatic disorders (Sdayria et al., 2018). Some molecules have the ability to inhibit phospholipase A₂ (PLA₂), cyclooxygenase (COX) and lipoxygenase (LOX) leading to a reduction in the production of prostaglandins (PGs) and leukotrienes (LTs) and inflammatory antagonism (Yahfoufi et al., 2018). However, their long-term therapeutic use is often associated with adverse effects such as gastrointestinal upset due to altered protective gastric mucosa (Wallace and Vong, 2008), renal damage and respiratory depression as well as possible dependence particularly with opioid analogues (Abiye et al., 2019). Some of drugs use ATP-sensitive potassium channel pathway leading to long-term cancer (Sharmin et al., 2016). More and more, people are falling back on traditional medicine especially on medicinal plants for their phytochemicals an active area of research for their anti-inflammatory properties. *Balanites aegyptiaca* L.

Delille (Balanitaceae) is native of Arabia, India, ki, Latin America and North Africa and it colonized the Sahel and savannah of West and East Africa according to Boffa (1999). Different parts of the plant (fruits, seeds, barks and roots) are used in traditional medicine for the treatment of pathologies such as laxatives, stomachaches, sterility, mental diseases, epilepsy, yellow fever, syphilis and as a vermifuge, rheumatism, toothaches, jaundice, liver and spleen problems (Maydell, 1983; Tata Kadiatou et al., 2018). Several pharmacological activities have been developed with different parts of *Balanites aegyptiaca* around the world and more precisely in Burkina Faso where they have developed a galenic formulation of syrup against intestinal parasites (Salfo et al., 2018). The aim of this study is to evaluate the ability of ethanolic and aqueous root bark extract to inhibit the production of pro-inflammatory enzymes (phospholipase A₂, COX 1 and 2 and LOX), to evaluate activity of anti-edematous, analgesic activity and observe whether they use the ATP-sensitive potassium channel pathway.

MATERIALS AND METHODS

Chemicals

Carrageenan and acetylsalicylic acid were obtained from Sanofi Winthrop Industry (France). Acetaminophen, acetic acid, sodium tetraborate, boric acid come from Sigma (Saint Louis, USA). 1,2-diheptanoilthio-glycerophosphocholine (1,2dHGPC), secretory phospholipase A₂ (sPLA₂) from bee venom and 5,5'-dithiobis-2-nitrobenzoic acid (DTNB) were obtained from Cayman Chemical Co. (MI, USA). For the colorimetric inhibition of COX-1 and human COX-2, Screening Kit (Item No. 560131) manufactured by Cayman Chemical, USA, was used. Other products and reagents [methanol, dimethyl sulfoxide (DMSO), Solumedrol, 5-lipoxygenase (EC: 1.13.11.12) type 1-B (extracted from soybean 167 U / mL), (Prolabo), were also used.

Animals

NMRI male mice 1-2 months old weighing 25-30 g from the Institute of Research in Health Science (IRSS) pet shop was used for the acute toxicity studies. They were provided by the IRSS pet shop where they were fed wheat cake (29% protein) and running water. They were raised under air conditioning (23-25°C) and 60% humidity. All experiments were conducted in the morning in accordance with the Laboratory Animal Care Guidelines and the Ethical Guidelines for Painful Experimentation on Conscious Animals (Zimmermann, 1983).

Plants materials and extraction

The part of *B. aegyptiaca* L. Delille (Balanitaceae) were harvested

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in the locality of Ouagadougou in 2015. The plant was botanical identified by Professor Amado Ouédraogo (Laboratoire de Biologie et Ecologie Vegetale / University Joseph KI-ZERBO, Ouagadougou, Burkina Faso) and a specimen was deposited at the herbarium of the university against a code number T4263. Plant material was dried at room temperature, ground (mill with East Gladiator type blades) and stored in an airtight bag until use.

Fifty (50) mg of powder was mixed with 500 ml of distilled water and boiled under reflux for 30 min. After cooling, the mixture is centrifuged and the supernatant is frozen for lyophilization. Two hundred and fifty (250 g) of the powder were extracted by exhaustion with a mixture of ethanol: water (9: 1). Rotary vacuum evaporator was used to remove the solvent and extract was freeze dried. The lyophilizate obtained (26.5 g for ethanolic extract and 11.95 g for aqueous extract) was stored in a vacuum desiccator for later use.

***In vitro* anti-inflammatory activity**

Phospholipase A₂ inhibition test

sPLA₂ activity was determined using method of Cayman Chemical Co. (MI, USA) (D'Almeida et al., 2013). For the assay, the wells (enzyme test) received 10 µl of methanol (HPLC) plus 10 µl sPLA₂ and the inhibition wells received 10 µl of reconstituted sPLA₂ then 10 µl of extract (8mg / ml) or of the reference compound. The blank consisted of 10 µl of methanol (HPLC) with 10 µl of buffer. The reaction was initiated with the addition of 200 µl of Diheptanoythiol-PC substrate and each mixture is made of triplicate then the entire device is allowed to incubate for 15 min at 25°C. After 15 min, 10 µl of DTNB was added to each well to stop the reaction. Mixing of the plate was carried out for 1 min and the reaction mixture was analyzed spectrophotometrically (spectrophotometer BioRad model 680, Japan) at the wavelength of 405 nm. All tests were done in triplicate and the percent inhibition of sPLA₂ was calculated by the following formula:

$$\% \text{ Inhibition} = [(AEA - AIA) / AEA] \times 100$$

-AEA: Activity enzyme test absorbance;
Enzyme Activity Test: Abs Enzyme test - Blank abs
-AIA: activity inhibition test absorbance
Activity Inhibition Test: Abs Inhibition test - Blank Abs

Enzymatic cyclooxygenase (COX1 and COX2) inhibitory activity

The COX activity assay kit (Catalogue No. 560131, Cayman Chemical, Ann Arbor, MI, USA) measures the peroxidase activity of cyclooxygenase. The peroxidase activity is assayed colorimetrically by monitoring the appearance of oxidized N,N,N',N'-tetramethyl-p-phenylenediamine (TMPD) at 590 nm (Kulmacz and Lands, 1983). For the assay, in the reaction medium, the test enzyme batch consisted of 150 µl of assay buffer, 10 µl of heme and 10 µl of the COX 1 or COX 2 enzymes and 10 µl of the dimerization solvent (DMSO). The inhibition test batch consisted of 150 µl of assay buffer, 10 µl of heme, 10 µl of COX 1 or COX 2 enzymes and 10 µl of ethanolic and aqueous extracts of root bark (2.2 mg / ml) or positive control such as aspirin and indometacin. The blank consisted of 160 µl of assay buffer, 10 µl of Heme and 10 µl of dimerization solvent (DMSO). The mixture was shaken for a few seconds and incubated for 5 minutes at 25°C. Twenty microliter of colorimetric substrate was added to each well used and the reaction was initiated by adding 20 µl of arachidonic acid solution to all wells used. The plate was carefully dried for a few seconds to

mix and incubate for 2 min at 25°C. Absorbance was read at 590 nm using a plate reader (Epoch, BioTeck instruments, USA). All tests were done in triplicate. the percent inhibition of COX 1 or COX 2 was calculated by the following formula:

$$\% \text{ Inhibition} = [(AEA - AIA) / AEA] \times 100$$

-AEA: Activity enzyme test absorbance
Enzyme Activity Test: Abs Enzyme Test - Blank abs
-AIA: activity inhibition test absorbance
Activity Inhibition Test: Abs Inhibition Test - Blank Abs

Lipoxygenase (LOX) inhibitory activity

The inhibition of lipoxygenase was assayed according to the spectrophotometric method described by E Malterud and Rydland (2000) with slight modifications. Briefly, 100 µl of enzyme solution (200 U/ml) prepared in boric acid buffer (0.2 M; pH 9.0) was mixed with 25 µl of aqueous and ethanolic extracts of *B. aegyptiaca* (8 mg/ml in DMSO) and then incubated at room temperature for 3 min. Reaction was then initiated by the addition of 125 µl of the substrate (250 µM of linoleic acid) and the velocity was recorded for 3 min at 234 nm with a microplate reader (Epoch, BioTeck instruments, USA). All tests were done in triplicate and DMSO was used as a control while Zileuton, positive control, were used as reference compounds. Percentage of lipoxygenase inhibition was calculated according to the equation:

$$\% \text{ Inhibition of lipoxygenase} = [(V_0 \text{ Control} - V_0 \text{ Sample}) / V_0 \text{ Control}] \times 100$$

V₀ Control: Enzymatic activity without inhibitor, V₀ Sample: Enzymatic activity in presence of extract or reference compounds.

***In vivo* anti-inflammatory activity**

Anti-edematous activity

The experimental study of the anti-inflammatory activity was carried out according to the method described by Winter et al. (1962) slightly modified by Noufou et al. (2012) according to which the inflammation is induced by injection of carrageenin at the level of the plantar arch of the paw right of the rat. The edema caused by this carrageenin will be translated into volume and measured by the Plethysmometer (Ugo Basile) which makes it possible to follow the evolution of the inflammatory process. Six batches of seven mice used (weight between 20-30 g) were fasted 17 h before the test. The mice were grouped into:

Control group: The mice of this batch receive the vehicle solution (physiological saline) orally, 01 hour before the injection of 50 µl of carrageenin (1% in NaCl 0.9%) into the plantar arch of the right leg of the mouse.

Reference group: The mice in this batch were treated with acetylsalicylic acid (ASA) at a dose of 100 mg / kg bw, 1 h before the injection of carrageenan.

Tests group (04 groups): The extracts to be tested are administered to mice orally at doses of 100, 200, 400 to 600 mg / kg; 01h before the injection of carrageenan.

The volume of the treated paw was measured before 01 h, and at 01, 03 and 05 h after the carrageenan injection. The anti-inflammatory activity was evaluated as a percentage of edema reduction in treated mice compared to blank controls according to the following formula:

Table 1. Phospholipase A₂, cyclooxygenase (COX 1 and COX 2) and 15-lipoxygenase inhibition activity.

Enzyme	Phospholipase A2	COX 1	COX 2	LOX
%	Inhibition percentage (at 100 µg/ml)			IC ₅₀ µg/ml
Ethanollic extract	45.82±1.3	42.35±1	33.1±2	83.75±20.09
Aqueous extract	42.84±1.5	54.12 ±2	10.22±3	110.66±6.21
Indomethacin	nd	nd	58.71±0.41	nd
Zileuton	nd	nd	nd	2.92±0.24

$$\text{Inhibition \%} = ((A - B)/A) \times 100$$

A represents the average difference of the volume of increase of the paw of the mice of the blank control group at the times T 01, T 03 and T 05h and B represents the average difference of the volume of increase of the paw of the mice of the treated batches at the times T 01, T 03 and T 05h. These averages are plotted on a curve to follow the evolution of the edema for each group. The determination of the percentage of inhibition of edema (IOP) makes it possible to evaluate the anti-inflammatory potential of the extract studied and to compare it with that of the reference product.

Analgesic activity: Writhing test

The analgesic effect of the extracts was evaluated according to the number of abdominal contortions induced by the intraperitoneal injection of acetic acid (0.6%) according to the method described by Noufou et al. (2012). Lots of seven were randomly constituted. The white control group received distilled water, the reference group, acetaminophen (paracetamol) a dose of 200 mg / kg and the other lots received the aqueous and ethanollic extracts of the root rinds at doses ranging from 100, 200, 400 to 600 mg / kg. One hour after extracts or reference administration, the animals intraperitoneally received acetic acid 0.6% at the dose of 10 ml / kg. Five minutes after the injection of acetic acid, the number of contortions was counted in each mouse for 15 min. The analgesic effect was evaluated according to the following formula:

$$\text{Inhibition \%} = ((Nb - Nt)/Nb) \times 100$$

Nb is the average of the number of contortions of the mice of the blank control group and Nt is the average of the number of contorted mice of the batch treated.

Non-morphine analgesic activity: Involvement of ATP-Sensitive K⁺ Channel Pathway

The non-morphine analgesic activity involving the K⁺ channels was evaluated by the method described by Perimal et al. (2010), with slight modifications. Four groups of 7 mice were divided as follows: the control group receives only the vehicle (saline), the second group receives glibenclamide (an ATP sensitive K⁺ channel inhibitor) and the last two groups receive respectively the ethanollic and aqueous extracts at a dose of 400 mg / kg of body weight. All mice except the first were pretreated with glibenclamide (10 mg / kg) 15 min before administration of saline or both extracts. Mice were injected with acetic acid, 1 hour after treatment. Five minutes after the acetic acid injection the number of contortions was recorded for 15 min. The analgesic effect was evaluated according to the following formula:

$$\text{Inhibition \%} = ((Nb - Nt)/Nb) \times 100$$

Nb is the average of the number of contortions of the mice of the blank control group and Nt is the average of the number of contorted mice of the batch treated.

Statistical analysis

Experiments were carried out in triplicate and results expressed as mean ± SEM. The analysis of the results was done on the basis of statistical processing of Graph Prism software version 6 and One way Analysis of Variance followed by Dunnett's test was used as a statistical treatment. The differences were considered significant when $p \leq 0.05$ compared to the control.

RESULTS

***In vitro* anti-inflammatory activity**

The percent inhibition of the activity of the phospholipase A₂ enzyme was close to 50% for both extracts as indicated in the Table 1. The inhibition percentages of cyclooxygenases 1 and 2 gave results on both sides comparable to the control for COX 2. The percentage inhibition of COX 1 by the two extracts was close to 50% whereas for COX 2 this percentage is very low with the aqueous extract than that ethanollic (Table 1). The results of the IC₅₀ of the LOX are shown in Table 1. Of the two extracts, the ethanol extract has a lower IC₅₀ than the aqueous one even though both have a lower enzymatic activity than that of the reference compound the Zileuton.

***In vivo* anti-inflammatory activity**

Anti-edematous activity

The results of the anti-edematous activity of the two extracts are shown in Figures 1 and 2. The effect of carrageenan on the mice which received the vehicle led to an increase in the volume of the paw from the first hour and reached the maximum at the fifth hour Pretreatment with different types of extracts at doses of 100, 200, 400 and 600 mg / kg significantly reduced carrageenan-induced edema. The reduction of edema by the extracts was dose-dependent and their percent inhibition was nevertheless lower than that of the reference,

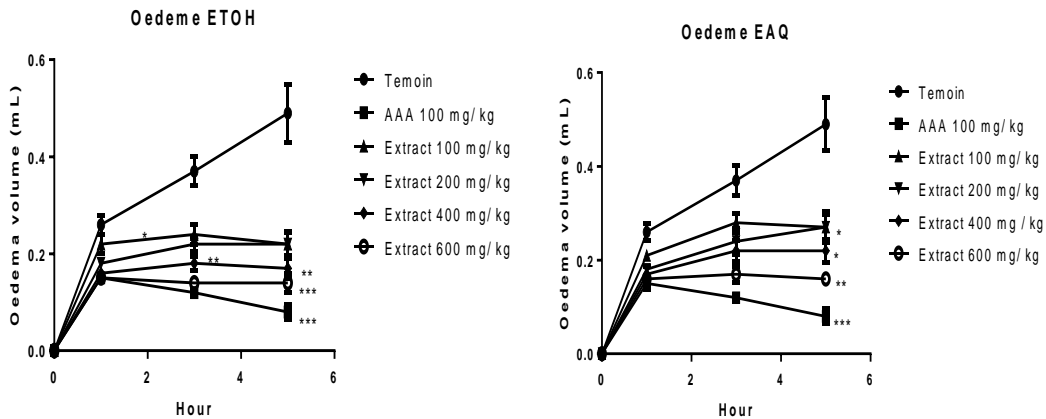


Figure 1. Effect of ethanolic (Oedema ETOH) and aqueous (Oedema EAQ) extracts at different doses on carrageenan-induced mouse paw edema. Values are mean \pm S.E.M. n = 7. *, **, *** indicate a significant difference respectively at $p < 0.05$, $P < 0.01$ and $p < 0.001$ compared to the control (ANOVA, post test Dunnett).

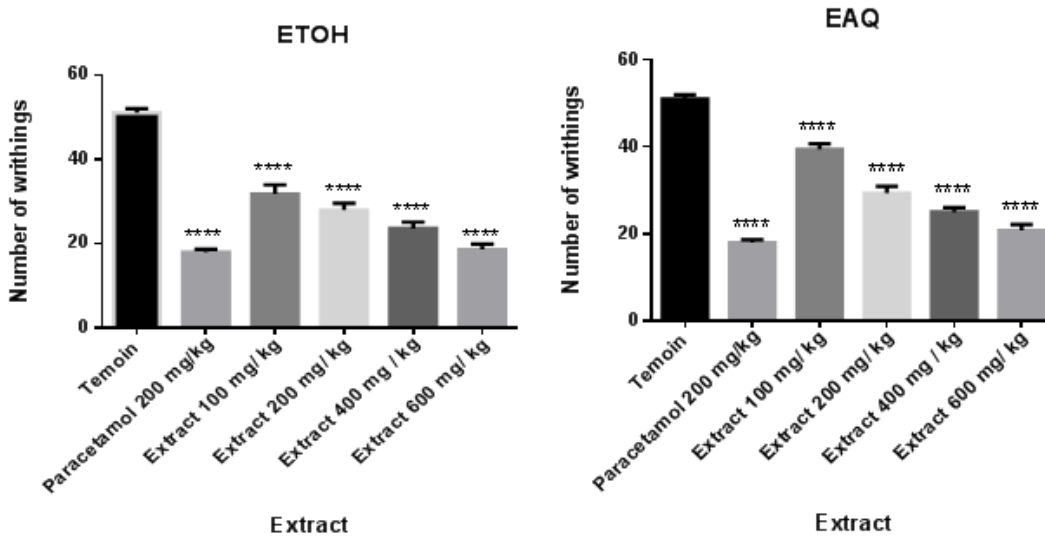


Figure 2. Effect of ethanolic (ETOH) and aqueous (EAQ) extracts on acetic acid induced writhing in mice. Values are mean \pm S.E.M. n = 7. ***: $P < 0.001$ indicate significance compared with control normal group (one way ANOVA analysis followed by Dunnett's test).

acetylsalicylic acid dosed at 100 mg/kg which was 83.56% at the fifth hour.

greater than 50% for the doses of 400 and 600 mg / kg compared with paracetamol.

Analgesic activity: writhing test

Figure 2 show the number of contortions due to the injection of acetic acid (0.6%). the number of fractions significantly decreased with the action of paracetamol at the dose of 200 mg / kg. the action of the extracts was dose-dependent and the percentage of the extracts was

Non-morphine analgesic activity: Involvement of ATP-Sensitive K^+ Channel Pathway

The writhing number of the mice receiving glibenclamide was comparable to that of the control mice that received the vehicle (Figure 3). Both extracts reduced the number of contortions by 50.98 and 53.73% respectively for the

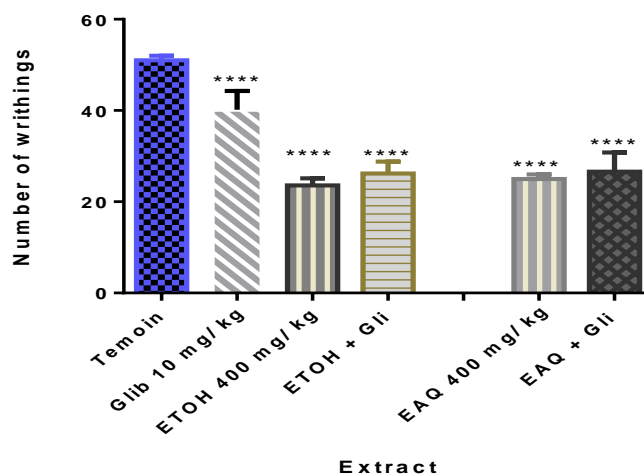


Figure 3. Effect of pretreatment of animals with glibenclamide (Glib) on the involvement of the K^+ channels of the ethanolic (ETOH) and aqueous (EAQ) extracts against the acetic acid-induced abdominal resistance test in mice. Values are mean \pm S.E.M. ***: $P < 0.05$ indicate significance compared with control normal group (one way ANOVA analysis followed by Dunnett's test).

aqueous extract and the ethanol extract in the absence of glibenclamide and in its presence its percentages decreased by less than 50% at 47.84 and 48.63% respectively. The action of glibenclamide increased the number of contortions.

DISCUSSION

Lipoxygenase enzymes (LOX) as well as cyclooxygenases, pro-inflammatory enzymes, are heavily involved in many diseases including atherosclerosis, cancer and diabetes (Vidal et al., 2007). Its enzymes cause the production of eicosanoids, which are the finished products of arachidonic acid metabolism. Phospholipase A₂ is a key enzyme in the metabolism of membrane phospholipids and its cellular stimulation is the crucial step in the production of pro-inflammatory mediators, prostaglandins (COXs) and leukotrienes (15-LOX) (Khanum et al., 2005). In this study, the enzymatic activity of the ethanolic and aqueous extracts of *Balanites aegyptiaca* root bark was demonstrated through the inhibition of Phospholipase A₂ enzymes, COX 1 and 2 and lipoxygenase *in vitro*.

Activity of phospholipase A₂ was inhibited by the extracts thus blocking the production of arachidonic acid. Extracts would be able to block phospholipases A₂ which of its activation is due by stimuli such as bradykinin, tumor necrosis factor interleukin-1 or calcium ionophore A23187 (Feghali-Bostwick and Wright, 1997).

COX1 is constitutive and plays a physiological role in

maintaining the integrity of tissues, while COX2 is inducible by inflammatory stimulation (Jouzeau et al., 1997). The extracts inhibited the activity of the COXs to different degrees. The extract acting on both COX 1 and COX 2, would have better anti-inflammatory and analgesic properties (COX 2) (Blobaum and Marnett, 2007; Burdan et al., 2004). These results are in agreement with those of Eldeen and Van Staden (2008) who in these studies found that the ethanolic extract of the roots of *Balanites aegyptiaca* inhibited the isoenzymes (COX-1 and 2).

According to Chung et al. (2009), it would be judicious that an extract having the inhibitory therapeutic effects of COX-1 and COX-2 be studied in the process of LOX inhibition it could be the only preferred route of conversion of arachidonic acid. in leukotrienes (leukotriene B₄, LTB₄) which play the major role in the inflammatory response. Both ethanolic and aqueous extracts of *B. aegyptiaca* showed moderate activity (41-70% inhibition) in the inhibition of 15-LOX according to the classification explained by Chung et al. (2009). The two extracts having an action on the inhibition of COX 1 and 2 enzymes and 5-LOX could have a synergistic effect in blocking the production of prostaglandins and leukotrienes (Irrera and Bitto, 2017). Both extracts inhibit the enzymatic activities of phospholipase A₂, COX 1 and 2 and 5-LOX at different percentages and at each stage of the inflammatory process so it would be a good anti-inflammatory to fight against inflammatory diseases (George et al., 2014).

The method of induction of edema by carrageenan was

used for inflammatory activity in vivo. According to Chatter and Tarhouni (2009) the method of induction of carrageenan would be simple, rapid in the induction of symptoms characteristic of inflammation (development of edema within one hour after injection, with an effect maximum after 5 hours) and reproducible. The induction of the inflammatory process by the carrageenan is manifested in 3 stages: The early stage (the first 90 min) during which histamine and serotonin are released; the second stage (90-150 min) which is driven by the release of kinin; and the third step (after 180 min), which is mediated by prostaglandin (Guo et al., 2011) (Rock et al., 2018).

B. aegyptiaca extracts significantly reduced ($p < 0.05$) carrageenan-induced edema in a dose-dependent manner by acting progressively on it with strong inhibition observed at the fifth hour. The action of the extracts would reduce the inflammation by inhibition of the enzyme cyclo-oxygenase responsible for the production of prostaglandins and thromboxane (Etamé Loé et al., 2018). Similar results have been found with different parts of the plant such as aerial parts (Gaur et al., 2008) and seeds (Ali et al., 2014) in reduction of edema.

The writhing test is occasionally classified as a visceral pain model, but both visceral and somatic structures are activated by intraperitoneal injection of acetic acid (Berge, 2011). The analgesic effect of *B. aegyptiaca* extracts was studied by inducing pain with acetic acid. This induced pain is thought to be due to the released chemical mediators (histamine, bradykinin, substance P and prostaglandins) that would stimulate peripheral nociceptive and induce an increase in vascular permeability (Noufou et al., 2012). This high vascular permeability leads to the formation of an exudate compressing the nerves at the origin of the sensation of pain (Etamé Loé et al., 2018). The action of the extracts reduced the number of contortions in a dose-dependent manner with a 50% inhibition with the 400 mg / kg dose with both ethanolic and aqueous extracts. The extracts would have an inhibitory effect on pro-inflammatory chemical mediators. The inhibitory effects of these extracts could be attributed to their inhibitory action on the enzymes involved in the synthesis of prostaglandins and leukotrienes.

This study also revealed the mechanisms including involvement of ATP-sensitive K^+ channels in the antinociceptive activities of *B. aegyptiaca* extracts. The single dose of 400mg / ml extracts that inhibited the number of contortions in the writhing test by 50% was used for non-morphine analgesic activity involvement of ATP-sensitive K^+ channel pathway. The action alone of glibenclamide (an ATP-sensitive potassium channel antagonist) did not significantly alter the number of contortions caused by the injection of acetic acid. The number of contortions significantly decreased the antinociceptive effects of *B. aegyptiaca* with injections of

extracts and glibenclamide (< 0.05). According to Nushrat and al., glibenclamide specifically blocks only drugs sensitive to ATP- K^+ channels, but does not affect other types such as Ca^{2+} activated and voltage-gated K^+ channels (Sharmin Ani et al., 2016). Scientific studies have shown that K^+ channels play a major role in pain, and that their opening causes a hyperpolarization of the cell membrane, leading to a decrease in cell excitability (Lawson, 2000). The blocking of K^+ channels did not have too much effect on the action of the extracts, which could be explained by the fact that the extracts do not follow the K_{ATP} path. Extracts do not seem to take the path of K_{ATP} which would mean that their long-term use will have virtually no carcinogenic effect on the body.

Conclusion

In conclusion, ethanolic and aqueous extracts of *B. aegyptiaca* root bark showed good inhibitory activity of pro-inflammatory enzymes such as phospholipase A2, COX 1 and 2 and 5-LOX. The extracts showed dose-dependent anti-inflammatory and analgesic activities in the different models used in this study and they do not seem to follow the K_{ATP} pathway. The present study could confirm the traditional uses of *B. aegyptiaca* against inflammatory pathologies.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Full Length Research Paper

Therapeutic effectiveness assessment of antiviral drugs used in chronic hepatitis treatment in three Ivorian university hospitals

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This work aims to evaluate the effectiveness of drugs used for the management of chronic viral hepatitis in the three major university hospitals in Abidjan. This is a retrospective cross-sectional descriptive study of 203 patients investigated for treatment protocols, patient parameters before and after treatment. It emerged that treatment with pegylated Interferon alpha 2a resulted in a significant decrease in viremia for an average treatment duration of 48 weeks in patients with chronic viral hepatitis B; on the one hand, the combination of Ribavirin + pegylated Interferon alpha 2a, Ribavirin + Sofosbuvir and Daclatasvir + Sofosbuvir provided cure in patients with chronic viral hepatitis C for an average duration of treatment of 12 to 24 weeks. However, the cure of patients with chronic viral hepatitis C was mostly clinical. The examinations that could attest to the healing were not performed by most of the patients, due to the high costs of virologic and enzymatic ones. These results constitute an important database for the therapeutic survey of people with chronic viral hepatitis in Côte d'Ivoire and an additional argument to accelerate the general subvention for the management of these viral diseases.

Key words: Viral hepatitis B-C-D, antiviral drugs, virologic markers, enzymatic markers.

INTRODUCTION

Chronic viral hepatitis remains a major global public health issue (Hutin et al., 2018). These infections due to hepatitis B (HBV) and hepatitis C (HCV) viruses mainly affect Africa: the prevalence of HCV in Central

Africa is estimated at 10%, and that of HBV in WestAfrica is estimated at 8% overall (Fera, 2015). Also, in Côte d'Ivoire, despite a prevalence of about 12% of HBV and 5% of HCV, detection and management of

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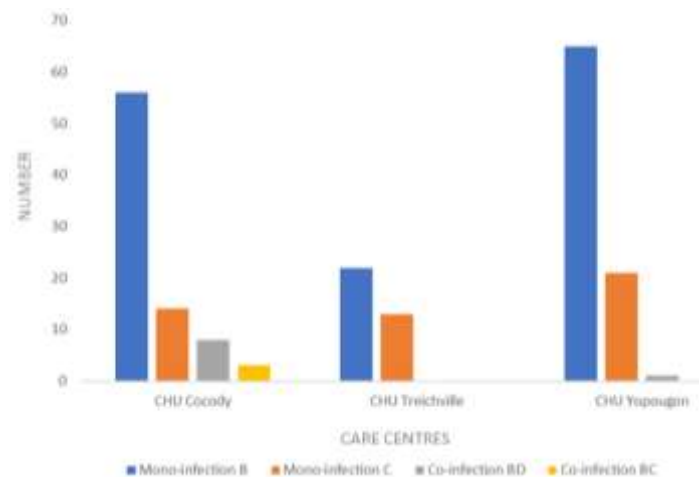


Figure 1. Distribution of study subjects according to viral type.

viral hepatitis B and C are still very limited (Enel et al., 2015) due to the costs of not only biological examinations but also of drugs. Indeed, the vial of pegylated Interferon alpha 2a, sufficient just for one week of treatment costs 300 US dollars, while treatment lasts for 24 to 48 weeks. In response to the threat posed by this scourge, Côte d'Ivoire has adopted, since 2015, a strategy to reduce mortality due to hepatitis B, C and delta (D) viruses by subsidizing some drugs, especially pegylated Interferon alpha 2a (Pegasys[®]) and Ribavirin (Copegus[®] and Ribavir[®]), indicated in the treatment of these viral infections in approved centers, namely three University Hospitals (CHU) in Abidjan; Bouaké in province and the Regional Hospital (CHR) in Yamoussoukro (PNLHV, 2017). The consequences of this strategy have not been assessed yet in terms of therapeutic service. Three years after the establishment of this national programme for the subsidized management of drugs for chronic viral hepatitis in Côte d'Ivoire, what is the therapeutic outcome? This is the research question that prompted the present investigations in the three main university hospitals in Abidjan, also the most frequented by patients receiving chronic viral hepatitis drugs. Thus, the objective of this study is to describe the treatment protocols established in Abidjan university hospitals, as well as the virologic and enzymatic parameters of patients before and after treatment.

MATERIALS AND METHODS

This study, not a clinical trial, but a retrospective, descriptive and cross-sectional study, was carried out on the basis of custom survey sheets for the patients' records, semi-direct interviews with physicians in charge of chronic viral hepatitis and telephone interviews with patients to collect some information missing from clinical records. It focused on patients with chronic viral hepatitis, registered in the databases of hospital pharmacies of the University Hospitals of Cocody, Treichville and Yopougan in Abidjan for the dispensing of medicines, and managed in hepato-gastroenterology units of the same hospitals from August 2015 to

July 2018.

The patients' questionnaire focused on the type of chronic hepatitis, the molecules of the treatment protocol, the duration of treatment and the results of biological examinations (Appendix).

The dispensing of the medicines was done exclusively in the pharmacies of approved centers outside tenofovir 300 mg tablets (Gentovir[®]) which was delivered in private pharmacies. In the pharmacy units of the university hospitals, medicines were the same, that is, Peginterferon 180 µg/mL vial (Pegasys[®]), Ribavirin 200 mg tablets (Copegus[®]) or 200 mg capsules (Ribavir[®]), Sofosbuvir 400 mg tablets (SSB[®]) and Daclatasvir 60 mg tablets (Dakasvir[®]).

Biological examinations were performed mainly at three centers, namely Pasteur Institute of Cocody, CIRBA (Integrated Bio-clinical Research Centre in Abidjan) and Longchamp laboratories as private center. The determination of viral load used molecular biology based on the principle of a nucleic acid amplification test for the quantitation of Hepatitis B Virus DNA and Hepatitis C Virus RNA genotypes 1 through 6 in human plasma and serum. For the other markers (HBsAg, anti-HBc, HBeAg, anti-HBe, anti-HCV), methods used were either enzyme immunoassay principle with formation of a sandwich complex. The results are determined automatically by the software by comparing the electrochemiluminescence signal obtained from the reaction product of the sample with the signal of the cutoff value previously obtained by calibration. This is either an enzyme-linked fluorescent immunoassay (ELFA) that is performed in the automated VIDAS system, or a combination of an enzyme immunoassay sandwich method with a final fluorescent detection, that is ELFA.

RESULTS

Viral types

During the study period, 203 patient records were found, the majority of which were mono-infection with viral hepatitis B (Figure 1).

Treatment regimens

Treatment protocols were essentially based on the combination therapy, in particular the bi-therapy

Table 1. Treatment regimens of chronic viral hepatitis in Abidjan University Hospitals.

Viral type	Molecules	Presentation	Dosage	Treatment duration
Mono-infection B	Peginterferon + Tenofovir	180 µg/mL vial	1 vial subcutaneous per week	48 weeks
		300 mg tablets	1 tablet in the morning	Seroconversion
Mono-infection C	Peginterferon + Ribavirin	180 µg/mL vial	1 vial subcutaneous per week	12 to 24 weeks
		200 mg tablets/capsules	≥ 75 kg: 1200 mg in two taken daily ; <75 kg: 1000 mg in two taken daily	
	Sofosbuvir + Daclatasvir	400 mg tablets	1 tablet in the morning	12 weeks
		60 mg tablets	60 mg in the morning	
Ribavirin + Sofosbuvir	200 mg tablets/capsules	≥ 75 kg: 1200 mg in two taken daily ; <75 kg: 1000 mg in two taken daily	12 to 24 weeks	
	400 mg tablets	1 tablet in the morning		
Co-infection BD	Peginterferon	180 µg/mL vial	1 vial subcutaneous per week	48 weeks
Co-infection BC	Sofosbuvir + Ribavirin + Peginterferon	400 mg tablets	1 tablet in the morning	12 weeks
		200 mg tablets/capsules	≥ 75 kg: 1200 mg in two taken daily ; <75 kg: 1000 mg in two taken daily	
		Ampoule 180 µg/ml	1 vial subcutaneous per week	

including almost totally pegylated Interferon alpha 2a (Table 1).

Virologic markers prior to treatments

The diagnosis of viral hepatitis B was made mainly by the determination of the HBs antigen (Table 2). HCV testing was conducted by detecting the antibody directed against HCV antigen. If positive, viral RNA was tested at a rate greater than 15 IU/L for chronic viral hepatitis C. HCV antigen determination was not necessary (Table 3).

Post treatments virologic markers

The HBs antigen and viral DNA were the most determined markers for evaluating the efficacy of viral

hepatitis B treatment (Table 4).

As with the diagnosis, testing for antibodies directed against HCV antigen and/or viral RNA were the markers for assessing the cure of viral hepatitis C (Table 5).

Enzymatic markers

Most of the subjects in our study series had enzymatic activities Alanine Aminotransferase (ALT) and Aspartate Aminotransferase (AST) varying between 30 and 60 IU/L prior to treatment (Table 6), to decrease from 10 to 30 IU/L after treatment (Table 7).

DISCUSSION

In carrying out our retrospective and cross-sectional

descriptive study on the evaluation of the effectiveness of pharmacological treatment, we noted that for the management of patients with chronic viral hepatitis B, the molecules used were pegylated Interferon alpha 2a associated with Tenofovir. Since Tenofovir is not included in the grant, patients purchased it from private pharmacies. Thus 143 patients with chronic viral hepatitis B were placed under this protocol, of which 46 patients were able to complete their treatments with pegylated Interferon alpha 2a, and only 6 patients were able to perform virologic examinations after treatment. Results of virologic examinations performed after the use of pegylated Interferon alpha 2a showed a significant decrease in viral level (undetectable viral load less than or equal to 20 IU/L) for 5 patients. Indeed, according to the World Health Organization (WHO), which recommends the treatment of viral hepatitis B

Table 2. virologic characteristics of patients with HBV prior to treatment.

Number	HBsAg	Anti-HBc	Anti-HBe	HBeAg	Viral DNA
26	+	+	ND	ND	ND
02	+	+	+	-	ND
14	+	+	-	-	ND
08	+	+	ND	ND	+
01	+	+	-	+	+
06	+	+	+	-	+
86	+	ND	ND	ND	ND
Total: 143					

+, Positive marker; -, negative marker; ND, not determined

Table 3. Virologic characteristics of patients with HCV prior to treatment.

Number	Anti-VHC	VHCaG	Viral RNA
16	+	ND	ND
32	+	ND	+
Total		48	

+, Positive marker; -, negative marker; ND, not determined.

Table 4. Post treatment virologic markers of patients with HBV.

Patients	HBsAg	Anti-HBc	Anti HBe	HBeAg	Viral DNA (UI/L)	Treatment duration
Patient 1	+	ND	ND	ND	-	48 weeks
Patient 2	+	ND	ND	-	+ ($\geq 56,64$)	24 weeks
Patient 3	+	ND	ND	ND	- (≤ 10)	144 weeks
Patient 4	+	ND	ND	ND	- (≤ 10)	48 weeks
Patient 5	+	ND	ND	ND	- (≤ 20)	20 weeks
Patient 6	+	ND	ND	ND	- (≤ 20)	48 weeks

+, Positive marker; -, negative marker; ND, not determined.

Table 5. virologic markers of patients with HCV declared cured after treatment.

Number	Anti-VHC	VHCaG	Viral RNA	Treatment duration
3	-	ND	Undetectable	12 and 24 weeks
14	ND	ND	ND	ND
Total 17				

-, Negative marker; ND, not determined.

Table 6. AST and ALT activities of subjects prior to treatment.

Activity (UI/L)	AST	ALT
10-30	15 patients	12 patients
30-60	17 patients	19 patients
60-90	01 patient	04 patients
> 90	01 patient	01 patient
Total	36 patients	36 patients

Table 7. Post-treatment AST and ALT activities.

Activity (U/L)	AST	ALT
10-30	9 Patients	8 Patients
30-60	6 Patients	5 Patients
60-90	0 Patient	2 Patients
> 90	0 Patient	0 Patient
Total	15 Patients	15 Patients

with Tenofovir or Entecavir, powerful molecules to significantly decrease viral load and rarely leading to the emergence of a pharmaco-resistance; the objective of the treatment is to achieve a profound suppression of viral multiplication reflected by circulating viral DNA (WHO, 2016). The French National Society of Gastro-Enterology (SNFGE), for its part, recommends two therapeutic strategies: the first, based on Interferon, aiming to obtain a prolonged virologic response after the cessation of treatment and the second, based on long-lasting nucleoside or nucleotide analogues (Entecavir, Tenofovir), usually lifetime, for a stable viro-suppression over time (SNFGE, 2015). In practice, the objective is the seroconversion of the HBe antigen (HBeAg) to anti-HBe antibodies in patients who are HBeAg positive, while in patients with chronic HBeAg negative hepatitis, the objective is to obtain a lasting viremia of less than 105 copies/ml (WHO, 2016). Thus, pegylated Interferon alpha 2a inhibits viral replication and promotes decreased viremia (Perrillo, 2006). Pegylated Interferon alpha 2a therefore remains effective in the management of patients with chronic viral hepatitis B (Fried et al., 2002). However, for a better evaluation of the effectiveness of treatments, it is important to introduce generic forms of Tenofovir systematically in hospital pharmacies of Abidjan university hospitals.

For chronic viral hepatitis C, 48 patients were recorded. The molecules used were pegylated Interferon alpha 2a associated with Ribavirin in 35 patients, Sofosbuvir associated with Ribavirin in 4 patients and Daclatasvir associated with Sofosbuvir in nine patients, with average treatment duration of 12 weeks to 24 weeks. The protocols were therefore based more on indirect-acting antivirals than on direct-acting antivirals, contrary to WHO recommendations. WHO and SNFGE recommend direct-acting antiviral treatment (Daclatasvir, Ledipasvir, Velpatasvir, Sofosbuvir, Simeprevir, etc.), all with combining, for some genotypes, pegylated Interferon alpha 2a and/or Ribavirin (SNFGE, 2015; WHO, 2014). At the end of these treatments, 17 patients were declared cured and only 3 patients were able to perform the paraclinical examinations. Biological evidence of healing has not often been established. However, it is apparent that treatment protocols for the management of patients with chronic C viral hepatitis are effective and provide healing, at least clinically. These results are consistent with studies that have shown an effectiveness of bi-

therapy «pegylated Interferon alpha 2a + Ribavirin» with 55% of the sustained virologic response rate (Fried et al., 2001). Our results are also consistent with those studies that have shown an effectiveness of bi-therapy «Sofosbuvir + Daclatasvir» with a sustained virology response greater than 80% (Hézode et al., 2015). Authors have also reported good results with other therapeutic combinations. For example, in patients treated with "Ledipasvir + Sofosbuvir" or "Ledipasvir + Sofosbuvir + Ribavirin", 90 to 100% achieved a sustained virologic response 12 weeks after discontinuation (Afdhal et al., 2014; Kowdley et al., 2014; Lawitz et al., 2014; Gane et al., 2014).

For BD and BC co-infections, only 12 patients were recorded, including 9 patients for HBDV and 3 patients for HBCV. Pegylated Interferon alpha 2a was the only molecule used to manage BD co-infection for an average treatment duration of 48 weeks, in accordance with WHO and SNFGE recommendations for the management of delta virus infection (WHO, 2016; SNFGE, 2015). The molecules used were Sofosbuvir, pegylated Interferon alpha 2a and Ribavirin for the management of co-infection BC for an average treatment duration of 12 to 24 weeks.

Virologic examinations were not performed by these patients for the maximum duration of treatment. Exchanges with them revealed a continuation of treatments with Tenofovir and pegylated Interferon alpha 2a.

Clinically, if the subsidized molecules (pegylated Interferon alpha 2a and Ribavirin) have been shown to be effective in the management of chronic viral hepatitis, the completion of paraclinical examinations remains a real problem for these patients, who find the costs very high. For example, some patients were forced to discontinue treatment, increasing the number of treatment failures due to lack of financial means, and others to the failure to perform their examinations, making it difficult to survey therapy. In this context, the effectiveness of treatments becomes clinical, and there are often cases of relapse since the paraclinical examinations; in this case the virologic examinations are the only ones that attest to the effectiveness of the treatments.

Conclusion

A descriptive retrospective study to assess the

effectiveness of the treatment of chronic viral hepatitis in Côte d'Ivoire was done. In six patients with a high viral load at the beginning of treatment, five had an undetectable viral load at the end of the treatment period with pegylated Interferon alpha 2a. As for the treatment protocols for the management of patients with chronic C viral hepatitis, they provided clinical healing, since biological evidence was established only for three patients. Faced with the barriers to effective treatment of viral hepatitis in our country, it is wise to focus action on awareness, prevention and early detection. Another means of control would be the systematic screening of pregnant women because they are the gateway to the screening of their entourage in case of positivity, and subsequent early management of detected-positive people and vaccination for detected-negative people. In addition, the extension of the grant to biological monitoring and other molecules becomes necessary.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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APPENDIX

Fact sheet

1) Patient identification

Record number _____

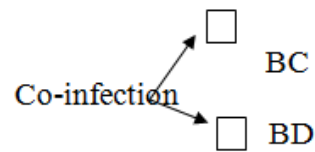
Patient name _____

Age: 0-14 years 15-24 years 25-50 years 51 years and older

Gender: male female

Care start: _____

Viral type: B C D



2) Treatment protocol

- Peginterferon
- Peginterferon + Tenofovir
- Peginterferon + Ribavirin
- Peginterferon + Sofosbuvir + Ribavirin
- Ribavirin + Sofosbuvir
- Sofosbuvir + Daclatasvir

3) Treatment duration

- 12 weeks
- 24 weeks
- 48 weeks
- Other to be specified _____

4) Biological examinations results**Serology:** (+) positive (-) negative

Laboratory: _____

Viral load before treatment.

Sampling date	HBsAg	Anti-HBs	Viral DNA	Anti-HBe	HBeAg	anti-HBC	Viral RNA

Viral load after treatment.

Sampling date	HBsAg	Anti-HBs	Viral DNA	Anti-HBe	HBeAg	anti-HBC	Viral RNA

5) Enzymatic activities

AST or ALT before treatment:

yes	no	unknown
<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>

○ AST _____ U/l

○ ALT _____ U/l

AST or ALT after treatment:

yes	no	unknown
<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>

○ AST _____ U/l

○ ALT _____ U/l

Full Length Research Paper

Non-adherence to statins therapy and its impact on cardiac morbidity and mortality: A metanalysis

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Dyslipidemia is a major risk factor for cardiovascular disease, the leading cause of death worldwide. Statins have been shown to significantly reduce morbidity and mortality in patients with coronary artery disease and in patients with hyperlipidemia. However, there is a significant gap between expected and actual benefits; this may be attributed to poor adherence to statin therapy. Literature search was conducted by using Pubmed, Wiley interscience, and EMBASE electronic databases for relevant studies for the meta-analysis. Inclusion criteria in this analysis were randomized controlled trials, retrospective analysis of data from randomized controlled trials, and observational studies. Adherence to statin therapy is suboptimal in both primary and secondary prevention of cardiovascular disease. The aim of this metanalysis was to assess non-adherence rates to statins in patients enrolled in both primary and secondary cardiovascular diseases prevention and to evaluate the impact of statins non-adherence over time on cardiac morbidity and mortality. Causes of non-adherence to statins are shown a discrepancy and include patient factors, practitioner factors and health system factors. Non-adherence is associated with adverse health outcomes and increased costs of health care. Non-adherence to statins is a significant issue for the prevention and treatment of cardiovascular disease. Increased awareness of the causes and solutions for overcoming non-adherence including safer prescribing, improvent in physician-patient alliance and reduction in drug costs, will enhance the cost-effectiveness of the use of statins and significantly improve patient care and outcomes.

Key words: Statins, non-adherence, cardiovascular diseases.

INTRODUCTION

Cardiovascular disease is the leading cause of death in the industrialized world. In developing countries there has also been a commensurate increase in the prevalence of this disease. Hydroxymethyl glutaryl-coenzyme A reductase (HMG_COA reductase) inhibitors (Statins) are

the most commonly prescribed medications for decreasing lipid levels. In 2005, 29.7% million individuals in the United States (US) were prescribed statin therapy (<http://en.wikipedia.Org/wiki/statin>).Largescale,clinicalend-point trials in a wide spectrum of subjects have

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demonstrated the universal efficacy of statins in the prevention of coronary, cerebrovascular and peripheral vascular disease in both primary and secondary prevention settings. In these trials, cholesterol lowering has been in the order of 20- 40%, with a commensurate relative risk reduction in clinical events. Despite their well-established benefits and corresponding recommendations from expert bodies, statins are widely underused in the 'real world' of clinical practice (Alsheikh et al., 2007; Kane and Lipsky, 2000; Hey-Hadavi et al., 2007). Studies also suggest, that patients' adherence to statins therapy is suboptimal and the persistence among those newly prescribed statins is poor. One study found that 40% of elderly patients lacked adequate statin therapy three months after receiving a prescription, and 60% lacked adequate supply after one year (Wolozin et al., 2007). Poor adherence to statin therapy is associated with adverse health outcomes, including higher hospitalization rates and increased non-pharmacy medical costs. Earlier studies have identified patient characteristics associated with statin therapy non-adherence, such as age, sex, comorbidities, and costs (Bates et al., 2009). There is a major gap between the use of statins in clinical trial settings and actual practice (LIPID, 1998). Unfortunately, little is known about suboptimal use of statins and its impact on cardiac morbidity and mortality. Therefore, the aims of this meta-analysis to assess non-adherence rates to statins in patients enrolled in both primary and secondary cardiovascular diseases prevention and to evaluate the impact of statins non-adherence over time on cardiac morbidity and mortality.

PHARMACOLOGY OF STATINS

Statins

The statins (or HMG-CoA reductase inhibitors) are a class of drug used to lower plasma cholesterol level. They lower cholesterol by inhibiting the enzyme HMG-CoA reductase, which is the rate-limiting enzyme of the mevalonate pathway of cholesterol synthesis. Akira Endo and Masao Kuroda of Tokyo, Japan commenced research into inhibitors of HMG-CoA reductase in 1971. This team reasoned that certain microorganisms may produce inhibitors of the enzyme to defend themselves against other organisms, as mevalonate is a precursor of many substances required by organisms for the maintenance of their cell wall (ergosterol) or cytoskeleton (isoprenoids) (<http://en.wikipedia.org/wiki/statin>). The first agent isolated was mevastatin (ML-236B), a molecule produced by the fungus *Penicillium citrinum*. The pharmaceutical company Merck & Co. showed an interest in the Japanese research in 1976, and isolated lovastatin (mevinolin, MK803), the first commercially marketed statin, from the fungus *Aspergillus terreus* (Ma et al.,

1986) (Figure 1).

Mechanism of action

Statins act by competitively inhibiting HMG-CoA reductase, the first committed enzyme of the HMG-CoA reductase pathway (<http://en.wikipedia.org/wiki/statin>). Because statins are like HMG-CoA on a molecular level they take the place of HMG-CoA in the enzyme and reduce the rate by which it can produce mevalonate, the next molecule in the cascade that eventually produces cholesterol, as well as several other compounds. This ultimately reduces cholesterol via several mechanisms (Figure 2) (Ma et al., 1986).

Inhibiting cholesterol synthesis

By inhibiting HMG-CoA reductase, statins block the pathway for synthesizing cholesterol in the liver. This is significant because most circulating cholesterol comes from internal manufacture rather than the diet. When the liver can no longer produce cholesterol, levels of cholesterol in the blood will fall. Cholesterol synthesis appears to occur mostly at night, so statins with short half-lives are usually taken at night to maximize their effect. Studies have shown greater LDL and total cholesterol reductions in the short-acting simvastatin taken at night rather than the morning, (Cilla et al., 1996, Ma et al., 1986) but have shown no difference in the long-acting atorvastatin (Ma et al., 1986).

Increasing LDL uptake

Liver cells sense the reduced levels of liver cholesterol and seek to compensate by synthesizing LDL receptors to draw cholesterol out of the circulation. This is accomplished via protease enzymes that cleave a protein called "membrane-bound sterol regulatory element binding protein", which migrates to the nucleus and causes increased production of various other proteins and enzymes, including the LDL receptor. The LDL receptor then relocates to the liver cell membrane and binds to passing LDL and VLDL particles (the "bad cholesterol" linked to disease). LDL and VLDL are drawn out of circulation into the liver where the cholesterol is reprocessed into bile salts. These are excreted, and subsequently recycled mostly by an internal bile salt circulation (Ma et al., 1986).

Other effects

Statins exhibit action beyond lipid-lowering activity in the prevention of atherosclerosis. The ASTEROID (A Study

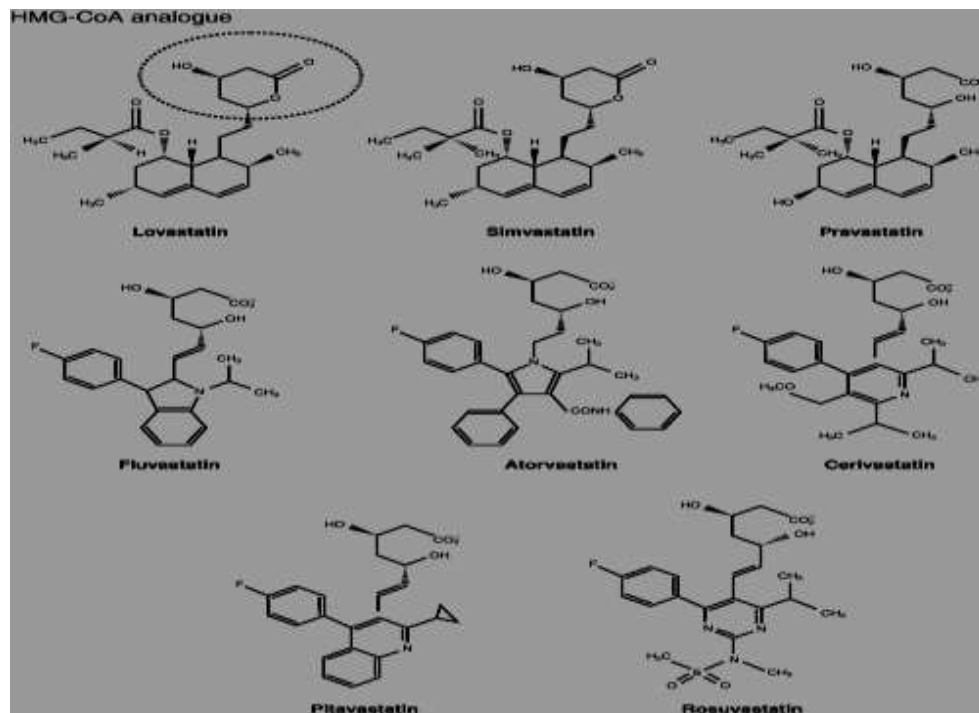


Figure 1. Chemical structures of the statins.
Source: <http://en.wikipedia.org/wiki/statin>

to Evaluate the Effect of Rosuvastatin on Intravascular Ultrasound-Derived Coronary Atheroma Burden) trial showed direct ultrasound evidence of atheroma regression during statin therapy (Nissen et al., 2006). Researchers hypothesize that statins prevent cardiovascular disease via four proposed mechanisms: improve endothelial function, modulate inflammatory responses, maintain plaque stability, and prevent thrombus formation (Furberg, 1999).

Statin may even benefit those without high cholesterol. In 2008 the JUPITER (Justification for the Use of Statins in Primary Prevention: An Intervention Trial Evaluating Rosuvastatin) study showed fewer stroke, heart attacks, and surgeries even for patients who had no history of high cholesterol or heart disease, but only elevated C-reactive protein levels. There were also 20% fewer deaths (mainly from reduction in cancer deaths) though deaths from cardiovascular causes were not reduced (Shear, 1992). Statins have been linked to a marked reduction in prostate cancer, benign prostate enlargement, incontinence and impotence in older men (Law et al., 2003).

Indications and uses

While statins are effective in decreasing mortality in those

who have had previous cardiovascular disease there is not a mortality benefit in those at high-risk but without prior cardiovascular disease (Law et al., 2003). Statins, the most potent cholesterol-lowering agents available, lower LDL cholesterol (so-called "bad cholesterol") by 1.8 mmol/L. This translates in a 60% decrease in the number of cardiac events (heart attack, sudden cardiac death, angina etc), and a 17% reduced risk of stroke (Law et al., 2003). They have less effect than the fibrates or niacin in reducing triglycerides and raising HDL-cholesterol ("good cholesterol"). Professional guidelines generally require that the patient has tried a cholesterol-lowering diet before statin use is considered; statins or other pharmacologic agents may then be recommended for patients who do not meet their lipid-lowering goals through diet and lifestyle approaches. The indications for the prescription of statins have broadened over the years. Initial studies, such as the Scandinavian Simvastatin Survival Study (4S), supported the use of statins in secondary prevention for cardiovascular disease, or as primary prevention only when the risk for cardiovascular disease was significantly raised (Wilson et al., 1998). Indications were broadened considerably by studies such as the Heart Protection Study (HPS), which showed preventative effects of statin use in specific risk groups, such as diabetics. The ASTEROID trial using only a statin at high dose, achieved lower than usual target calculated

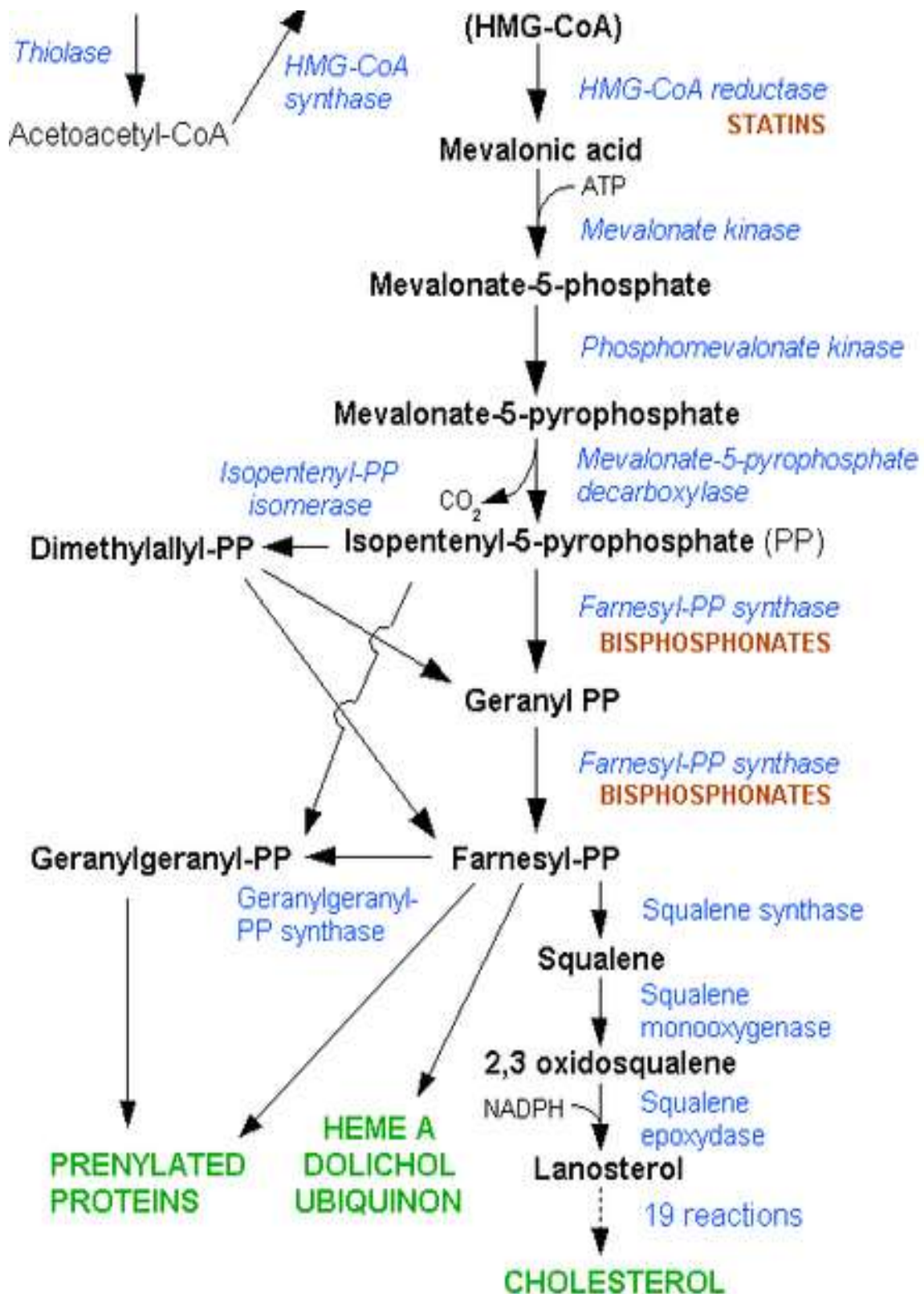


Figure 2. The cholesterol synthesis pathway, which is blocked by statins via inhibiting the rate limiting enzyme HMG-CoA reductase.

Source: <http://en.wikipedia.org/wiki/statin>

LDL values and showed disease regression within the coronary arteries using intravascular ultrasonography (Nissen et al., 2006).

Based on clinical trials, the National Cholesterol Education Program guidelines (NCEP), and the increasing focus on aggressively lowering LDL-cholesterol, the statins continue to play an important role in both the primary and secondary prevention of coronary heart disease, myocardial infarction, stroke and peripheral artery disease (Furberg, 1996).

Members

Fermentation-derived and synthetic

The statins are divided into two groups: fermentation-derived (naturally occurring) and synthetic. The synthetic ones include Atorvastatin, Cerivastatin, Fluvastatin Rosuvastatin and Pitavastatin. LDL-lowering potency varies between agents. Cerivastatin is the most potent, followed by (in order of decreasing potency), rosuvastatin, atorvastatin, simvastatin, lovastatin, pravastatin, and Fluvastatin (Ryan, 2015). The relative potency of pitavastatin has not yet been fully established (Shepherd et al., 2008). Some types of statins are naturally occurring such as Lovastatin, Mevastatin, and Simvastatin. They can be found in such foods as oyster mushrooms and red yeast rice (Liu et al., 2006). Randomized controlled trials found them to be effective, but the quality of the trials was low (Amarenco et al., 2006).

Comparative effectiveness

No large-scale comparison exists that examines the relative effectiveness of the various statins against one another for preventing hard cardiovascular outcomes, such as death or myocardial infarction (Amarenco et al., 2006).

An independent analysis has been done to compare atorvastatin, pravastatin and simvastatin, based on their effectiveness against placebos. It found that, at commonly prescribed doses, there are no statistically significant differences amongst statins in reducing cardiovascular morbidity and mortality (Amarenco et al., 2006). The comparative dose efficacy study of atorvastatin versus simvastatin (CURVES) study, which compared the efficacy of different doses of atorvastatin, simvastatin, pravastatin, lovastatin, and fluvastatin for reducing LDL and total cholesterol in patients with hypercholesterolemia, found that atorvastatin was more effective without increasing adverse events (Jones et al., 1998).

Statin equivalence

Statins differ in their ability to reduce cholesterol levels. Doses should be individualized according to patient characteristics such as goal of therapy and response (Jones et al., 1998). After initiation and/or dose changes, lipid levels should be analyzed within 1-3 months and dosage adjusted accordingly, then every 6-12 months afterwards (Dotani et al., 2000, Pan et al., 2004) (Table 1).

Safety

Adverse effects

Statins are generally perceived as well-tolerated. The most common adverse side effects are raised liver enzymes and muscle problems. In clinical trials, reported adverse effects are low; but "higher in studies of real-world use" and more varied. Statins increased the risk of an adverse effect by 39% compared to placebo (odds ratios 1.4); two-thirds of these were myalgia or raised liver enzymes with serious adverse effects like placebo (Abramson and Wright, 2007).

Some patients on statin therapy report myalgias, muscle cramps, or, less frequently, gastrointestinal or other symptoms. Liver enzyme derangements may also occur, typically in about 0.5%, are also seen at similar rates with placebo use and repeated enzyme testing, and generally return to normal either without discontinuance over time or after briefly discontinuing the drug (Abramson and Wright, 2007). Multiple other side-effects occur rarely; typically, also at similar rates with only placebo in the large statin safety/efficacy trials. Two randomized clinical trials found cognitive issues while two did not; recurrence upon reintroduction suggests that these are causally related to statins in some individuals. One Danish study suggested a relation between long term statin use and increased risk of nerve damage or polyneuropathy (Golomb and Evans, 2008), but suggested this side effect is "rare, but it does occur"; other researchers have pointed to studies of the effectiveness of statins in trials involving 50,000 people which have not shown nerve damage as a significant side effect (Silva et al., 2006).

More serious but rare reactions include myositis and myopathy, with the potential for rhabdomyolysis (the pathological breakdown of skeletal muscle) leading to acute renal failure. Coenzyme Q10 (ubiquinone) levels are decreased in statin use; Q10 supplements are sometimes used to treat statin-associated myopathy, though evidence of their effectiveness is currently lacking (Silva et al., 2006). A common variation in the *SLCO1B1* gene, which participates in the absorption of statins, has been shown to significantly increase the risk of myopathy

Table 1. Statin equivalence.

Statin equivalent dosages						
%LDL Reduction (approx.) (%)	Atorvastatin (mg)	Fluvastatin (mg)	Lovastatin (mg)	Pravastatin (mg)	Rosuvastatin (mg)	Simvastatin (mg)
20-Oct	-	20	10	10	-	5
20-30	-	40	20	20	-	10
30-40	10	80	40	40	5	20
40-45	20	-	80	80	10-May	40
46-50	40	-	-	-	20-Oct	80
50-55	80	-	-	-	20	-
56-60%	-	-	-	-	40 mg	-
Starting dose						
Starting dose	10–20	20	10-20	40	10 mg; 5 mg if hypothyroid, >65 yo, Asian;	20 mg
If higher LDL reduction goal	40 mg if >45%	40 mg if >25%	20 mg if >20%	-	20 mg if LDL >190	40 mg if >45%
Optimal timing	Anytime	Evening	With evening meals Anytime	Anytime	Anytime	Evening

Source: "http://en.wikipedia. Org/wiki/statin"

(Julie and steve 2002).

Graham et al. (2004) reviewed records of over 250,000 patients treated from 1998 to 2001 with the statin drugs atorvastatin, cerivastatin, fluvastatin, lovastatin, pravastatin, and simvastatin. The incidence of rhabdomyolysis was 0.44 per 10,000 patients treated with statins other than cerivastatin. However, the risk was over tenfold greater if cerivastatin was used, or if the standard statins (atorvastatin, fluvastatin, lovastatin, pravastatin, simvastatin) were combined with fibrate (fenofibrate or gemfibrozil) treatment. Cerivastatin was withdrawn by its manufacturer in 2001 (Teresa et al., 2006).

All commonly used statins show somewhat similar results, however the newer statins, characterized by longer pharmacological half-lives and more cellular specificity, have had a better ratio of efficacy to lower adverse effect rates. The risk of myopathy is lowest with pravastatin and fluvastatin probably because they are more hydrophilic and as a result have less muscle penetration. Lovastatin induces the expression of gene atrogen-1, which is believed to be responsible in promoting muscle fiber damage (Teresa et al., 2006).

Despite initial concerns that statins might increase the risk of cancer, various studies concluded later that statins have no influence on cancer risk (Teresa et al., 2006). Indeed, a 2005 trial showed that patients taking statins for over 5 years reduced their risk of colorectal cancer by 50%; this effect was not exhibited by fibrates. The trialists warn that the number needed to treat would approximate

5000, making statins unlikely tools for primary prevention. However, in a recent meta-analysis of 23 statin treatment arms with 309,506 person-years of follow-up, there was an inverse relationship between achieved LDL-cholesterol levels and rates of newly diagnosed cancer that the authors claim requires further investigation (Graham et al., 2004).

Drug interactions

Combining any statin with a fibrate, another category of lipid-lowering drugs increases the risks for rhabdomyolysis to almost 6.0 per 10,000 person-years. Most physicians have now abandoned routine monitoring of liver enzymes and creatine kinase, although they still consider this prudent in those on high-dose statins or in those on statin/fibrate combinations, and mandatory in the case of muscle cramps or of deterioration in renal function (Teresa et al., 2006).

Consumption of grapefruit or grapefruit juice inhibits the metabolism of statins. Furanocoumarins in grapefruit juice (that is, bergamottin and dihydroxybergamottin) inhibit the cytochrome P450 enzyme CYP3A4, which is involved in the metabolism of most statins (however it is a major inhibitor of only lovastatin, simvastatin and to a lesser degree atorvastatin) and some other medications (Graham et al., 2004). This increases the levels of the statin, increasing the risk of dose-related adverse effects (including myopathy/rhabdomyolysis). Consequently,

Table 2. Adherence observed during principal randomized controlled trials of statins for primary and secondary prevention of cardiovascular disease.

Trial	Author, year	Definition of adherence	Method of ascertainment	Mean lowering of serum cholesterol concentration (%)	Primary or secondary prevention	Mean age	Observation period (years)	Adherence at end of period (%)
AFCAS/ texCAS	JAMA, 1998	≥75%	Pill counts	19	Primary prevention	58	5.2	99
EXCEL	Shear et al. (1992)	≥75% medication taken	Self report	Not stated	Secondary prevention	54	4	99
CARE	Sacks et al. (1996)	Continuation of therapy	Not stated	20	Secondary prevention	59	Median 5	94
4-S	Conroy et al. (1998)	Continuation of therapy	Not stated	26	Secondary prevention	58	Median 5.4	90
Heart Protection Study	Farmer et al. (2003)	≥80% medication taken	Pill counts	17	Secondary prevention	64	Median 5	82
LIPID	The LIPID Study Group, 1998	Continuation of therapy	Not stated	18	Secondary prevention	62	6.1	81
WOSCOPS	Shepherd et al. (1995)	≥75%	Pill counts	20	Primary prevention	55	Mean 4.9	70

consumption of grapefruit juice is not recommended in patients undergoing therapy with most statins. An alternative, somewhat risky, approach is that some users take grapefruit juice to enhance the effect of lower (hence cheaper) doses of statins. This is not recommended as a result of the increased risk and potential for statin toxicity (Teresa et al., 2006).

MATERIALS AND METHODS

Search strategy

Searches for relevant research reports were conducted using Pubmed, Wiley interscience and EMBASE electronic data bases. The key words used for search were statins, HMG-COA reductase inhibitors, adherence, non-adherence, compliance and concordance. A manual search of the reference lists from retrieved paper was also performed to identify further relevant studies.

Inclusion criteria

Eligible for inclusion criteria in this analysis were randomized controlled trials, retrospective analysis of data from randomized controlled trials, and observational studies evaluating the association between adherence to statins therapy and its effect on cardiovascular diseases.

Data extraction

Parameters extracted from the studies included study design, number of patients, mean age of patients, mean study length,

definition of adherence.

Definition of nonadherence

Adherence (synonymous with compliance and concordance) is the 'extent to which patients follow the recommendations by their healthcare professional'. 'Adherence is the product of a relationship that is built on respect, active participation, and partnership between patient and health professional, not coercion or manipulation, on the part of either' (Marquez et al., 1998). Non-adherence is the inverse or reciprocal of adherence. Non-adherence can be subdivided into primary non-adherence (that is, the failure to initiate therapy) and secondary non-adherence (that is, the failure to continue therapy). Secondary non-adherence can further be categorized into failure to take the medication as directed (dose and frequency of regimen) and the premature discontinuation of the medication (Mahler et al., 1999). Most studies cited in this article have used a definition of adherence as a patient taking at least 80% of the prescribed doses, although some studies have used qualitative descriptions of adherence.

RESULTS

Study characteristics

Relevant research papers were identified from the literature search. From these papers studies which satisfied the inclusion criteria were identified and included in a meta-analysis. Table 2 display Adherence observed during principal randomized controlled trials of statins for primary and secondary prevention of cardiovascular

Table 3. Studies of statin adherence in the community setting.

Authors, year	Study design	Operational definition of adherence	Method of ascertainment	Primary or secondary prevention	Mean participant age	Observation period	Adherence at end of period (%)
Ho et al. (2006)	Cohort	Continuation of therapy	Structured telephone interview	Secondary prevention	65	6 months	87
Kotseva et al. (2009)	Cohort	≥80% medication availability	Pharmacy records	Secondary prevention	67	2.4 years	64
Bouchard et al. (2007)	Nested case-control	≥90% prescriptions filled	Data retrieved from healthcare database	Primary prevention	63	1 year	62
Law et al. (2003)	Retrospective cohort study	≥80% medication availability	Pharmacy records	Primary and secondary prevention	58	2 years	58
Bates et al. (2009)	Retrospective cohort study	300/365 minimum daily doses received	Health Authority database	Primary and secondary prevention	≥40	3 years	52
Vinker et al. (2008)	Retrospective cohort study	≥80% prescriptions filled	Pharmacy records	Secondary prevention	58	5 years	49
Benner et al. (2004)	Retrospective cohort study	Continuation of therapy	Pharmacy records	Primary and secondary prevention	60	3 years	21

disease. Adherence to statin therapy in clinical trials (Table 2) tends to be much higher than in every day practice (community settings) (Table 3).

Frequency of non-adherence

Non-adherence to statins is a surprisingly common problem. The exact rate of non-adherence is difficult to determine. However, clinical trial and post marketing data underscore the scale of the problem. In the West of Scotland Coronary Prevention Study (WOSCOPS), 6595 men with moderate hypercholesterolemia were randomized to pravastatin 40 mg a day or placebo. After a mean follow-up of 4.9 years there was a highly statistically significant reduction in coronary events of 31% (Bates et al., 2009). The authors correctly used an intention-to-treat analysis to show this benefit. However, the magnitude of benefit might have been higher, for at the first follow-up visit only approximately 85% of patients were adherent to treatment (WOSCOPS, 1997). A Canadian study of primary prevention cases reported high discontinuation rates for statin usage of 35 and 65% at 6 months and 3 years, respectively (Vinker et al., 2008). Retrospective data from a UK electronic database of 6462 diabetic patient records indicated that adherence to statin therapy was only 87% at 3 months, falling to 61% at 6 months and thereafter remaining stable over a follow-up period of 13 years. Only 50% of patients were fully adherent to the prescribed regimen, with the

remainder having some degree of non-adherence (Benner et al., 2002). In an Israeli study of 47,680 Health Management Organisation (HMO) patients there was a high rate of discontinuation at 12 months, with only 61% of patients adherent with 80% of their statin therapy; at 6 years of follow-up this had fallen to just 10% (Vinker et al., 2008). Elderly patients are a group who may be at particular risk of statin non-adherence. There are several reasons for this. These include polypharmacy, susceptibility to drug side effects, cognitive dysfunction, physical disability (poor eyesight, arthritis) and depression. In a retrospective cohort study of 34,501 patients over the age of 65 years enrolled in a Medicaid program, adherence to statin therapy was 79% at 3 months, falling to only 42% at 10 years (Benner et al., 2002). Significantly, after 5 years, only 25% of patients were adherent to prescribed statin therapy at least 80% of the time. Contrary to expectations, adherence to statins is also a significant problem in patients who have suffered a primary coronary event. Ho et al. (2006) retrospectively evaluated 13,596 patients with previous symptomatic myocardial infarction or coronary revascularization. Non-adherence, defined as a patient taking less than 80% of the prescribed medication, was assessed at 180 days for statins, beta blockers and ACE inhibitors: the rate of non-adherence was 26, 28 and 21% respectively. The Global Reduction in Acute Coronary Events (GRACE) investigators followed up acute coronary syndrome patients 6 months after discharge and reported lower rates of non-adherence than other

Table 4. Reasons for medication non-adherence.

Categories of Nonadherence	Examples
Health system (Hermans et al., 2010)	Poor quality of provider-patient relationship; poor communication; lack of access to healthcare; lack of continuity of care
Condition (Jackeviticus et al., 2008)	Asymptomatic chronic disease (lack of physical cues); mental health disorders (e.g., depression)
Patient factors (Ho et al., 2006)	Physical impairments (e.g., vision problems or impaired dexterity); cognitive impairment; psychological/behavioral; younger age; nonwhite race
Therapy (Thiebaud et al., 2005)	Complexity of regimen; side effects
Socioeconomic (Jackeviticus et al., 2008, Pletcher et al., 2009, Stranberg et al., 1994, LIPID, 1998)	Low literacy; higher medication costs; poor social support

studies (statins 13%, beta blockers 12%, ACE inhibitors 20%) (Ho et al., 2006). In a separate study, patients who were reviewed within 3 months of initiation of statin therapy were 45% more likely to be adherent than those who were not reviewed (Benner et al., 2004). Stroke patients have also been found to have a relatively high discontinuation rate for statins of 39% at 12 months compared with 17% for antiplatelet agents and 10% for ACE inhibitors. The discontinuation period for statin therapy varies over time. A retrospective cohort study of 239,911 patients who failed to refill a prescription for a statin found that 54% of patients had a period of discontinuation that lasted at least 90 days, with 48% of these patients resuming therapy within 1 year and 60% within 2 years (Colivicchi et al., 2007).

Risk factors for statin non-adherence

There are several causes of non-adherence; while some are obvious, some are more subtle and elusive. Identifying the causes of non-adherence is essential for managing patients. The factors that can influence non-adherence to statin therapy are presented in Table 4. In the WOSCoPS study, the predictors of non-adherence to pravastatin were smoking, younger age and the absence of hypertension (WOSCoPS, 1997). In the study by Ho et al. (2006) younger patients, those with depression and those with chronic obstructive pulmonary disease were more likely to be non-adherent to all classes of cardiovascular medications (including statins); there was also a nonsignificant trend towards increased non-adherence in those with dementia (Colivicchi et al., 2007). Whilst the young may be non-adherent owing to lack of concern regarding their health, the elderly are also at risk of non-adherence. In a study from the USA by Benner et al., older age, lower income and depression were predictors of non-adherence to statins.

Furthermore, the presence of cardiovascular disease also predicted adherence; patients with a recent myocardial infarction were more likely to be adherent than patients whose prescription was for primary prevention. Jackeviticus et al. (2008) reported similar findings in a Canadian study. In this study the significant predictors of non-adherence with statins were younger age, low income status, pre-discharge counselling, and drug initiation by a cardiologist; diabetics also tended to be non-adherent. A separate study from the USA also suggested that poor literacy was a predictor of statin non-adherence (Pletcher et al., 2009). In a UK study, the predictors of non-adherence among diabetic patients included previous cardiovascular disease and older age (Amarenco et al., 2006). In contrast to the USA data, social disadvantage was not found to predict statin non-adherence in this group of diabetics. Those who had a new event on statin therapy were more likely to become non-adherent, suggestive possibly of disillusionment with the therapy (Vinker et al., 2008). A recently reported study of 6276 Belgian subjects found in a multivariate model that statin adherence as well as a positive patient outlook was significant factors in achieving LDL-C goals. The cost of medications remains a controversial cause of non-adherence. The largest series to evaluate the cost of medicines and adherence found that in 132 studies an increase in cost sharing was associated 'with lower rates of drug treatment, worse adherence among existing users, and more frequent discontinuation of therapy' (Hermans et al., 2010). The authors found that for a 10% increase in cost sharing, medication use falls by 2-6%. Data from a separate HMO study indicated that for a US \$10 increase in monthly copayments, there is a 1.8% reduction in statin adherence for those beginning therapy, and a 3% reduction in those continuing therapy (Goldman et al., 2006). Patients' non-adherent to statin therapy had more hospital visits and higher health care costs, although the cost difference between the groups did not

reach statistical significance. A recent study by Doshi et al. suggested that, for US Veterans Affairs patients, a rise in the copayment from US \$2 to \$7 was associated with a 7% fall in adherence rates, and a 12% increase in discontinuation rates lasting 90 days or more (Doshi et al., 2009). There are other factors that may lead to statin non-adherence.

In an HMO study, Sung et al. (1998) found that in women, multiple doses of lipid-lowering therapy and overall good health were predictors of medication nonadherence, suggesting that polypharmacy in those without cardiovascular disease may fail owing to patients not realizing the need for therapy. Dormuth et al. (2009) recently reported that those adherent to statins were more likely to undergo screening tests, and were less likely to be involved in accidents, suggesting that patients who are adherent may be more health conscious and more risk averse. Switching statins has also been shown to increase the likelihood of non-adherence (Thiebaud et al., 2005). A study of patients who switched from one statin to another found that 'switchers' were 19% less adherent to their statin than 'non-switchers'. Cost was not considered to be a reason for switching or nonadherence (cost difference between groups US \$1.33/month). The reason for switching statins was not elucidated and may have been the same reason for discontinuation (e.g., side effects, lack of perceived benefit, patient perception) (Thiebaud et al., 2005) (Table 4).

Statin adverse effects and non-adherence

The side effects of statins experienced by patients are also an important cause of non-adherence to medication. In this context, the best evidence of statin discontinuation rates was reported in the Prediction of Muscular Risk in Observational Conditions (PRIMO) study (Bates et al., 2009). In this observational study, 7,394 French patients with dyslipidemia were studied to determine the rates and predictors of muscle side effects from statins. Overall 19.8% of subjects discontinued their statin therapy, whilst 16.7% required a dose reduction. Most of the muscle side effects occurred within the first 3 months, like the findings of Colivicchi et al. (2007). Predictors of discontinuation included a personal history of muscle pain on lipid-lowering therapy, unexplained cramps, a raised creatinine kinase, a family history of muscle symptoms with or without lipid-lowering therapy, and hypothyroidism. The more potent longer-acting statins (atorvastatin and simvastatin) had higher rates of discontinuation than the less potent pravastatin and fluvastatin XL. A recent study found that a small percentage of patients were unable to tolerate high doses of simvastatin because of a genetic polymorphism in an anion transporter protein (SLCO1B1) responsible for the hepatic uptake of statins; this impairment presumably led to higher systemic levels

of simvastatin and muscle toxicity (SEARCH, 2008). Interestingly, those patients with the genetic polymorphism were able to tolerate lower doses of simvastatin. Finally, no reason may be identified for non-adherence other than disillusionment with 'too many pills'. In the Colivicchio et al. stroke study, 72% of patients and their medical practitioners could not identify a medical reason for discontinuing statin therapy other than 'too many pills', with the other 28% discontinued for mild side effects [Colivicchi et al. (2007)].

Elevated alanine transaminase or aspartate transaminase levels were more common in patients treated with atorvastatin 80 mg compared with placebo (3.2 versus 0.9%), but specific musculoskeletal or liver abnormalities remained rare ($\leq 3\%$). A similar study involving a pooled analysis of 3145 patients aged ≥ 75 years who received placebo or atorvastatin 10-80 mg in 45 completed randomized trials demonstrated that the rate of adverse events did not increase with higher doses of the drug and was similar in atorvastatin-treated patients and those who received placebo. Thus, currently available evidence suggests that the safety of statin therapy remains similar in older and younger patients, even with intensive lipid lowering (Thiebaud et al., 2005) (Table 5).

Impacts of statin non-adherence on cardiac morbidity and mortality

Health consequences of statin non-adherence

Non-adherence to statins is associated with significant health risks. In the WOSCoPS study adherence was also found to be an independent predictor of adverse cardiovascular outcomes. In those who were adherent to pravastatin less than 75% of the time, the event rates for coronary death or nonfatal myocardial infarction were like placebo, whilst those who were adherent to pravastatin 75% or more of the time had significant reductions in these end points (WOSCoPS, 1997). In a separate nested case-control study of 20,543 primary prevention patients, it was found that non-adherence in the first year of therapy had little impact on the prevalence of nonfatal myocardial infarction; however, in those with adherence rates of less than 90% after 1 year, there was an excess risk of nonfatal myocardial infarction. Similar findings were recently reported in large database analysis, only 55% of patients were adherent to at least 80% of their statin doses. Those exhibiting the highest levels of adherence ($> 80\%$) had a 26% reduction in stroke compared with those who exhibited the lowest rates of adherence ($< 20\%$ adherence). Following a coronary event, non-adherence to statins is also associated with excess risk of a recurrent clinical event (Bouchard et al., 2007). In the study by Ho et al. (2006), patients with

Table 5. Adverse events reported in trials comparing different intensities of statin therapy among patients with coronary artery disease.

Variable	PROVE IT-TIMI 22		A TO Z		TNT		IDEAL		REVERSAL		SAGE	
	Low n=2063	High n=2099	Low n=2230	High n=2263	Low n=5006	High n=4995	Low n=4449	High n=4439	Low n=327	High n=327	Low n=445	High n=446
Event												
Adverse event leading to drug discontinuation, no (%) of patients	56(2.7)	69(3.3)	191(8.6)	216(9.5)	265(5.3)	360(7.2)	186(4.2)	426(9.6)	22(6.7)	21(6.4)	46(10.3)	48(10.8)
Aminotransferase level elevations, no. (%) of patients	23(1.1)	69(3.3)	8(0.4)	19(0.9)	9(0.2)	60(1.2)	5(0.1)	43(1.0)	5(1.6)	7(2.3)	1(0.2)	19(4.3)
Myalgia, no.(%) of patients	56(2.7)	69(3.3)	35(1.6)	50(2.2)	234(4.7)	241(4.8)	51(1.1)	97(2.2)	12(3.4)	9(2.8)	12(2.7)	14(3.1)
Myopathy (myalgia with creatinine elevation), no.(%) of patients	NR	NR	1(0.04)	9(0.4)	NR	NR	11(0.3)	6(0.1)	NR	NR	NR	NR
Rhodomlyolysis, no.(%) of patients	0	0	0	3(0.1)	3(0.06)	2(0.04)	3(0.07)	2(0.05)	0	0	0	0

NOTE: PROVE IT_TIMI 22= Pravastatin or Atorvastatin Evaluation and Infection Therapy - Thrombolysis in Myocardial Infarction 22, A-to-Z= Aggrastat to Zocor, TNT= Treating new targets, IDEAL= The Incremental Decrease in Events through Aggressive Lipid Lowering, REVERSAL=Reversal of atherosclerosis with aggressive lipid lowering therapy, SAGE=study assessing goals in elderly, NR=not reported.

lower rates of adherence had higher rates of all-cause mortality (non-adherence with statins OR 1.82; 95% CI 1.61- 2.06), cardiovascular hospitalizations (non-adherence with statins OR 1.35; 95% CI 1.21-1.51), and revascularizations (non-adherence with statins OR 1.11; 95% CI 1.01- 1.22) (Winland et al., 2000). Jackevicus et al. (2008) also reported that in postmyocardial infarction patients, those who did not take any of their discharge medications had an 80% increase in mortality at 12 months compared with fully compliant patients; those who were partially compliant with their medications had a 44% increase in mortality. The IDEAL investigators (Holme et al., 2009) recently reported that the benefit of high-dose atorvastatin versus simvastatin in postmyocardial infarction patients may have been underestimated owing to an excess of non-adherence in the atorvastatin group. Adjusting for adherence, the benefit in the

atorvastatin group increased to 15% from 11%, reaching statistical significance. Moreover, those adherents to either statin had significant reductions in cardiovascular and non-cardiovascular end points, suggesting that those who were adherent gained significant health benefit, and may have been more health conscious. In Kulikl et al. (2008), study of patients discharged from the hospital after the coronary artery bypass graft (CABG) surgery, demonstrated that statin therapy initiated with one month of CABG discharge is independently associated with a lower risk of all-cause mortality and major adverse cardiovascular events (MACE) even after adjustment for patient, hospital, and surgeon characteristics. These results support existing practice guidelines and confirm that in the absence of serious contraindications, essentially all patients should be prescribed long term statin therapy after CABG. Several studies have demonstrated that

preoperative statin therapy improves clinical outcomes after CABG, including a reduced risk of death, myocardial infarction, and arrhythmias in the first 60 days after surgery (Dotani et al., 2008; Pan et al., 2004). Statin therapy initiated within the first few months after hospital discharge independently reduces all cause mortality and MACE after CABG. Statin therapy provides high levels of protection for all cause mortality and non-hemorrhagic strokes (Hey-Hadavi et al., 2007). The stroke prevention by Aggressive Reduction in cholesterol levels (SPARCL) trial demonstrated that high-dose atorvastatin reduced the risk of subsequent stroke in patients with transient ischemic attack (TIA) or stroke and the absence of coronary artery disease patients in the SPARCL study randomized to high dose atorvastatin had significantly lower rates of stroke (RR 0.85, 95% CI, 0.73 -0.99), stroke or TIA (RR 0.77, 95% CI 0.67-0.88), and coronary events (0.65, 95% CI,

0.49-1.87) than placebo treated patients (Amarenco et al., 2006). A spate of recent clinical trials using statins to lower low-density lipoprotein cholesterol (LDL-C) have demonstrated beyond reasonable doubt that coronary events both morbid and mortal, can be prevented (WHO, 2009). The mean reduction in total cholesterol, LDL-C, and triglyceride levels was -20, -28, and -13% respectively, and HDL-C was increased by an average of 5% among the 5 trials included in meta-analysis (Bates et al., 2009). A meta-analysis from 5 clinical trials demonstrated that a significant reduction in the odds of major coronary events and coronary deaths ($p < 0.001$) was observed among the participants allocated to active treatment. The reduction in coronary events was 31% (95% CI), 26-36% and the reduction in fatal coronary disease was 29% (95% CI, 20-36%) (Kulik et al., 2008) (Tables 6 and 7).

Economic costs of statin non-adherence

The direct medical costs and indirect costs due to lost productivity from coronary heart diseases (CHD) in the United States are estimated to exceed \$142 billion in 2006 (Gibson et al., 2006). Recent evidence has demonstrated that patient financial incentives (i.e. co-payments, coinsurance) also affect statin adherence (Strandberg et al., 39). As statin cost sharing levels increase, adherence to statins falls. Patient cost-sharing also has been demonstrated to be a financial barrier to the utilization of other classes of medication that are typically used to treat chronic disease (Gibson et al., 2006; Goldman et al., 2006).

Peterson and McGhan have suggested that for patients who were 'almost 100% compliant with statins versus those with initial non-adherence the cost per life year saved (LYS) was US \$4,500 to > \$250,000 depending on patient age, presence or absence of risk factors and whether the statin is being used for primary or secondary prevention'. An intervention study suggested that the cost per patient to improve statin adherence for 1 year was approximately US \$154-279 (Peterson and McGhan, 2001). A separate study showed that the lower drug costs of non-adherence with lipid lowering therapy were far outweighed by the excess costs of increased cardiovascular disease (Gibson et al., 2006). A separate study modeled the effect of increasing the cost of copayments for statins in those at low risk of cardiovascular disease and reducing or abolishing the copayments for statins for those at high risk of cardiovascular disease (Goldman et al., 2006). The analysis concluded that such a change would increase adherence, and in such groups of patients would lead to 79,837 fewer hospitalizations and 31,411 fewer presentations to emergency departments, with projected

savings of US \$1 billion annually. Finally, a recent study by Pletcher et al. (2009) concluded that full implementation of the ATP III guidelines would require 11 million Americans to initiate or intensify statin therapy; in doing so 20,000 myocardial infarctions and 10,000 deaths would be avoided at a cost of US \$42,000/QALY. Though these costs were dependent on the cost of statin therapy, at lower medication prices the economic benefits became significantly more cost effective (Goldman et al., 2006).

Health psychology perspective

In addition to the effects of depression, several other psychological issues may bear on a patient's adherence behavior. Patient adherence is now viewed as the consequence of a complex interaction that involves numerous patient variables, effectiveness of physician communication, and the quality of the doctor-patient relationship during the medical consultation. Patient-centered care is more than just interviews with empathy; it requires the use of skills and tools that maximize a health care partnership and shared management of a chronic condition (Hermans et al., 2010). The psychological contributors to patient adherence behaviors are highly interactive and can be considered from the perspective of the patient, the doctor's perspective and the doctor-patient relationship. From the patient's perspective, issues such as satisfaction, health beliefs, and preferences for health care, can all influence intentional non-adherence. A patient's view on treatment, as measured by the Beliefs about Medicines Questionnaire (BMQ), can determine their adherence behaviour and provides valuable information for a doctor attempting to address patient concerns (Hermans et al., 2010). One study further proposes that social cognitive theory outlines a core set of determinants, one of which is perceived self-efficacy, that influence the adherence behaviour of an individual patient. Adherence self-efficacy is the belief in one's ability to organize and perform behaviors that are necessary to achieve one's health goals (Thiebaud et al., 2005). Several health psychology studies have found that adherence self-efficacy is associated with adherence to therapy and better use of health-related coping strategies (Molloy et al., 2008). Additionally, personality and cognitive function research into health behaviors has provided some evidence to support the possibility that conscientiousness and IQ can predict adherence behaviour to cholesterol-lowering treatment (Stilley et al., 2004). Furthermore, social support may also be a factor in patient adherence (Molloy et al., 2008). The recently developed Adherence Estimator measures three proximal patient beliefs associated with intentional non-adherence to new medications. Preliminary psychometric evidence

Table 6. Overall risk reductions of major coronary events and Deaths from coronary diseases, cardiovascular, and all causes trials.

Variable	No of events		Relative risk reduction,% (95% CI)	Absolute risk reduction 1000 (95%)	P value
	Placebo	Statin			
Major coronary events	2042	1490	31 (26-36)	36(29-43)	<0.001
4s, (Strandberg et al.,1994)	622	431	38(29-46)	86(61-111)	<0.001
WOSCops, 1997	248	174	31(16-44)	23(11-34)	<0.001
CARE (Klein et al., 2006)	274	212	25(10-38)	30(10-49)	0.002
AFCAPS/TexCAPS (Downs et al., 1998)	183	116	38(21-50)	20(10-30)	<0.001
LIPID, 1998	715	557	25(16-34)	35(21-50)	<0.001
Coronary events	748	543	29 (20-36)	13(9-18)	<0.001
4s	189	111	43 (38-55)	35(20-50)	<0.001
WOSCops	52	38	27 (-10-52)	4(1-10)	0.13
CARE	119	96	20 (-5-39)	11(-2-25)	0.11
AFCAPS/TexCAPS	15	11	27 (-58-66)	1(-2-4)	0.44
LIPID	373	287	25 (12-36)	19(8-30)	<0.001
Cardiovascular deaths	868	646	27(19-34)	14(10-19)	<0.001
4s ,	207	136	36(20-49)	32(16-48)	<0.001
WOSCops	73	50	32 (3-52)	7(0-14)	0.03
CARE	130	112	15 (-11-34)	9(-5-23)	0.23
AFCAPS/TexCAPS	25	17	32(-25 -63)	2(-1-6)	0.22
LIPID	433	331	25(14-36)	23(11-34)	<0.001
Non cardiovascular deaths	429	400	7(-7-19)	2(-2-6)	0.29
4s ,	49	46	6(-41-38)	1(-7-10)	0.760
WOSCops	62	56	10 (-29-38)	2(-5-8)	0.57
CARE	66	68	-3 (-45-27)	-1 (-12-10)	0.87
AFCAPS/TexCAPS	52	63	-21(-76-16)	-3(-10-3)	0.30
LIPID	200	167	17(-2-33)	7(-1-160)	0.08
All cause deaths	1297	1046	21(14-28)	16(11-22)	<0.001
4S	256	182	31(16-44)	33(16-51)	<0.001
WOSCoPs	135	106	22(0-40)	9(0-18)	0.05
CARE	196	180	9(-12-26)	8(-10-25)	0.38
AFCAPS/texCAPS	77	80	-4(-43-24)	-1(-8-6)	0.81
LIPID	633	498	24(14-33)	30(17-44)	<0.001

indicates that the best predictors of adherence behaviour are the patient's perceived need for medications, their perceived concerns about medications and their perceived affordability of medications (Thiebaud et al., 2005). From the physician's perspective, communication is an important component of patient care (Bates et al., 2009). Evidence suggests that physicians poorly predict patient adherence. Indeed, unintentional non-adherence may reflect an inadequate understanding on the part of the patient, of the condition, treatment, or prevention regimen prescribed and may be averted with enhanced communication by the physician. Improving provider-patient communication can have beneficial effects on health outcomes (Thiebaud et al., 2005) and it is important that physicians attend to both the cognitive and emotional care of their patients if optimal treatment

adherence is to be achieved (Fuentes et al., 2007). This emotional aspect is important and much of the literature on health management suggests that service providers with high emotional intelligence receive higher patient satisfaction scores (Weng, 2008). The value of emotional intelligence as a useful concept in patient-centered care is still being ascertained, but it may provide an explanation of why some practitioners are more successful in achieving higher rates of adherence to therapy in their patients.

The final contribution to the triad that influences patient adherence behaviour is the relationship between the patient and the physician. The quality of this interaction, referred to by Fuentes et al. (2007) as the physician-patient working alliance, has been recently studied using the Working Alliance Inventory (WAI). The results of this

Table 7. Overall risk reduction for major coronary events by sex and age: results from 5 randomized controlled.

Variable	No. of events		Relative risk reduction,% (95% CI)	Absolute risk reduction 1000 (95%)	P value
	Placebo	Statin			
Sex					<0.001
Women	247	180	29(13-42)	33(13-52)	0.01
4S (Strandberg et al., 1994)	91	60	37(10-56)	69(17-122)	0.04
CARE(Klein et al., 2006)	39	23	43(3-66)	54(4-104)	0.17
AFCAPS/TexCAPS (Downs et al., 1998)	13	7	46(-31-78)	12(-5-29)	0.30
LIPID, 1998	104	90	15(-15-37)	18(-16-51)	<0.001
Men	1795	1320	31(26-35)	37(29-44)	<0.001
4S	531	371	38(28-47)	90(62-118)	<0.001
CARE	235	189	22(5-36)	26(5-47)	0.02
AFCAPS/TexCAPS	170	109	37(20-50)	22(10-33)	<0.001
LIPID	611	467	27(17-36)	39(23-55)	<0.001
Age					<0.001
≥ 65y	740	539	32(23-39)	44(30-58)	<0.001
4S	168	122	38(19-53)	98(43-154)	<0.001
CARE	111	69	42(20-57)	65(27-103)	0.01
AFCAPS/TexCAPS	112	78	32(23-39)	44(30-58)	0.001
LIPID	349	270	25(11-37)	42(17-67)	<0.001
< 65 y	1302	951	31(24-36)	32(24-40)	<0.001
4S	454	309	38(27-47)	83(55-110)	<0.001
CARE	163	143	14(-9-32)	14(-8-37)	0.21
AFCAPS/TexCAPS	71	38	47(22-63)	19(8-31)	0.001
LIPID	366	287	25(12-37)	31(13-48)	<0.001

study provide preliminary evidence of a correlation between a patient's rating of this alliance and their adherence behaviour and suggest that these interpersonal dynamics seem to have 'real value' and are likely to make a difference in medical care (Fuentes et al., 2007).

Management of statin non-adherence

Improving statin adherence

Improving statin adherence is likely to lead to a reduction in cardiovascular end points and health care costs. Osterberg and Blacshke (2005) described four methods that may improve adherence. These include patient education and support, improved dosing schedules, increased availability of medical staff and improved communication between physicians and patients. Patient education is essential. It is well acknowledged that the level of awareness in the community as to what the desirable levels of cholesterol are is poor; in Northern Europe only approximately 1/2 of respondents could identify the normal level of plasma cholesterol. Patient awareness of cholesterol levels varies considerably;

those with higher educational levels and coronary artery disease are more likely to have undergone cholesterol testing and to know their level (Erhardt and Hobbs, 2002). Moreover, many patients with hypercholesterolemia will be asymptomatic and as such may not perceive the need to take medications. In an intervention in Spain with tutorials and postal follow-up questionnaires, adherence was 32.7% (81 vs 61%) higher in the intervention group than those not receiving the intervention (Cannon et al., 2004). A recent study from the UK found that patients given a brief counselling session at statin initiation followed by mailed education were 10% more likely to fill a statin prescription at 4 months than patients who followed usual care (Sprafka et al., 1989). Despite these positive studies, a recent study of 8104 statin-treated patients who were randomized to either usual care or an adherence-enhancing program for 12 months reported no difference in the achievement of target LDL-C between the two groups. These divergent results highlight the complex nature of achieving optimal compliance. Multiple authors have also suggested using simple, nontechnical and jargon-free explanations to communicate the benefits of therapy (Marquez et al., 1998). Moreover, it is essential to understand a patient's anxieties about therapy and to support their efforts to

improve adherence. This can be achieved by improving the patient–physician working alliance and by focusing on the psychodynamics of the interaction between patient variables and physician’s cognitive and emotional skills (Mahler et al., 1999).

In a clinic-based study from North Carolina, intervening with a multidisciplinary team in patients with abnormal lipid profiles led to significant improvements in medication adherence, plasma cholesterol levels and attainment of LDL-C goals compared with no intervention (Thomas et al., 2003). Similar improvements in health outcomes including medication adherence, risk factor modifications and dietary improvements were seen in a large intervention study in Europe in patients with established vascular disease (Fulmer et al., 1999).

Statins are prescribed once daily, but potency varies. Rosuvastatin and atorvastatin have the longest plasma half-lives; this potentially allows the medication to be taken with other morning medications, rather than at night as simvastatin and pravastatin must be. Combination therapy of a statin plus another medication in a single tablet (e.g., Simcor, simvastatin/ niacin combination; or a statin and antihypertensive medication, e.g., Caduet, atorvastatin/amlodipine) has been introduced and may improve adherence. Finally, memory aids for patients such as dosette boxes and alarms may also prove useful (Friedman et al., 1996). Prescribing a statin in hospital has been shown significantly to improve adherence and reduce mortality. In a study of 600 patients with angiographically proven coronary artery disease, those given inpatient statin therapy had significantly higher adherence (77 vs 40%; $p < 0.0001$) and lower mortality (5.7 vs 11.7%; $p = 0.05$) at follow-up than those prescribed statins in primary care (Fonarow et al., 1997). The GRACE investigators also reported that there was a significantly higher likelihood of patients taking their aspirin if prescribed by a cardiologist (Ho et al., 2006). A structured in-hospital prescribing program such as the Cardiovascular Hospitalization Atherosclerosis Management Program (CHAMP) has been shown to improve the implementation of evidence-based risk-reduction strategies as well as improving long-term medication adherence and reducing morbidity (Muhlestein et al., 2001). As indicated earlier, the presence or absence of cardiovascular disease is an important determinant of statin adherence. Some practitioners have advocated the use of imaging to improve medication adherence in those without symptomatic cardiovascular disease. In a study of 505 patients followed for a mean of 3.6 years, Kalia et al. (2006) used coronary computerized tomography angiography and calcium score to determine the presence or absence of coronary disease. In those with a low coronary calcium score (0- 99) adherence rates were 44- 63%, whilst in those with high scores (> 400) adherence rates reached 90%. This approach is invasive, costly and exposes the patient to ionizing

radiation, and as such has limited use.

However, it does suggest that other imaging modalities costly and exposes the patient to ionizing radiation, and as such has limited use.(e.g., carotid ultrasound for intima-medial thickness, plaques or stenosis) may be able to achieve similar results. Medical staff attitudes and availability are also important in managing patients with poor adherence to statins. The institution of evidence-based practice at the time of a cardiovascular event in hospital also increases adherence. Patients who default on appointments are also likely to be non-adherent to medication and are a group who would benefit from targeted education and support. Medical practitioners also need to recognize medication compliance as a major clinical problem. Several studies have shown that 40-66% of practitioners may not routinely ask patients about medication compliance; appropriate education of practitioners may improve this gap (Wilson et al., 2007; Allen et al., 2002). Physicians should consider routine screening for non-adherence in their clinical practice, using for example the Adherence Estimator for patients placed on new treatments, to target patients at risk of non-adherence. For reliable estimates of non-adherence and to reduce the effect of social desirability bias, patients should self-complete this predictive tool rather than have it directly administered in an interview format by a health care provider (Osterberg and Blaschke, 2005). Improved interprofessional communication involving the physicians, nurses and other allied health staff, and patients is also essential. The use of a qualified nurse who is adequately resourced has been shown to be cost-efficient, especially for smoking cessation. A prospective randomized intervention study in 2002 reported that the use of nurse practitioners following myocardial infarction resulted in a greater number of patients achieving the ATP III target LDL level than those randomized to usual care (Wilson et al., 2007). The use of advanced practice nurses for follow-up rather than physicians at the Cleveland Clinic has resulted in a reduction in LDL-C of approximately 0.9 mmol/L from baseline, as well as being positively received by patients (>83% of responses were positive) (Benner et al., 2004). The use of a dedicated pharmacy program may also achieve significant improvements in medication adherence and desirable reductions in LDL-C. Two hundred patients taking at least four chronic medications were enrolled in a 6-month interventional study, followed by half the patients being randomized to continued intervention or usual care. The intervention included medication education, regular pharmacist follows up and the dispensing of time-labeled medication packs. Following the initial intervention, adherence to lipid-lowering therapy rose from 61 to 97% with a fall in LDL-C of 9.5%. Following the randomization phase, those in the intervention group maintained high levels of adherence (96%), whilst in those receiving usual care adherence fell

Table 8. Significant pharmacological interactions with statins that may cause clinical myopathy and lead to non-adherence.

Interacting medication	Statin - cytochrome (Cyp) P450 metabolic enzyme				
	Simvastatin	Atorvastatin	Fluvastatin	Rosuvastatin	Pravastatin
	Cyp 3A4	Cyp 3A4	Cyp 2C9 Cyp 2C	Cyp 2C9 (min)	Non-Cyp metabolism
Cyclosporine A	+	+	-	+	+
Azole antifungals	+	+	-	-	-
HIV protease inhibitors	+	+	-	-	-
Macrolide antibiotics	+	+	-	-	-
Non-dihydropyridine calcium channel antagonists	-	-	-	-	-
Amiodarone	-	+	-	-	-
Antidepressants	+	+	-	-	-
Gemfibrozil	+	+	-	+	+

Source: Modified from Bates et al. (2009).

to 69% (Bellosta et al., 2004).

Therapeutic options for the statin-intolerant patients

Once a patient has discontinued statin therapy owing to an adverse effect, resuming therapy is important as there is substantial health benefit to be gained. In all patients with statin intolerance there must be a careful search for potential interactions (Table 8) (Bellosta et al., 2004), including interactions with other lipid-lowering therapies such as gemfibrozil, as well as with antibiotics (especially macrolides), immunosuppressive agents (especially cyclosporine A and tacrolimus) and protease inhibitors. Choosing an agent that is metabolized by a different cytochrome P450 isoenzyme than the statin in question can effectively guide therapy. Medical conditions that predispose to statin intolerance include excessive alcohol consumption, hypothyroidism, malnutrition and renal and hepatic dysfunction. Correction of these medical problems or dose reduction may improve statin tolerability. Asian patients can be prone to myopathy with conventional doses of statins and should always be initiated at the lowest prescribable dose (Bellosta et al., 2004); this is especially important with rosuvastatin, which should be prescribed at an initial dose of 5 mg daily (Schachter, 2005). In those patients in whom intolerance is the reason for non-adherence, a comprehensive multi-disciplinary plan should be implemented to manage this intolerance. Initially an alternative statin should be tried. This may include substitution of one statin for another or nonconventional dosing regimens. Substituting one statin for another may be effective owing to differences in physicochemical and pharmacodynamic properties. Longer-acting statins such as simvastatin and atorvastatin are lipophilic and have active metabolites (Stein et al., 2008). Rosuvastatin has a long plasma half-

life, but minimal active metabolites. These statins and their respective metabolites may accumulate in muscle and brain, leading to toxicity. Substitution of these statins with a statin undergoing extensive first pass metabolism, such as fluvastatin, or with a statin not dependent on cytochrome p450 metabolism, such as pravastatin, or a less lipid-soluble statin (such as pravastatin and rosuvastatin), may be effective in reducing toxicity (Bellosta et al., 2004). Should this be ineffective, then an alternative regimen can be used; several strategies have been reported and should be considered. A regimen of ezetimibe or fluvastatin XL or a combination of fluvastatin XL and ezetimibe therapy was trialed in 199 patients who had previously been statin intolerant due to muscle toxicity. After a period of 12 weeks, only 10 patients discontinued therapy due to muscle toxicity; each of the therapeutic options was equally well tolerated. In each of the groups there were significant improvements in plasma cholesterol and apolipoprotein B concentrations. Significantly, 80% of the combination therapy group reached their NCEP (National Cholesterol Education Program) cholesterol target levels. In a retrospective analysis, another group reported that alternate day therapy with rosuvastatin (mean dose 5.6 mg) was tolerated by 73% of patients previously intolerant to statins due to muscle toxicity (Ruisinger et al., 2009). A reduction in LDL-C of 35% was observed. Similar findings were recently reported from a retrospective analysis of 50 patients previously intolerant of statins due to muscle toxicity; these patients were able to tolerate a mean dose of 10 ± 4 mg a week of rosuvastatin. Finally, a cyclical regimen of dosing followed by a drug holiday may be effective in reducing symptoms. It has been suggested that Vitamin D deficiency increases the risk of statin myotoxicity, but this notion has not been tested in a clinical trial (Young et al., 2007). Some practitioners advocate the use of coenzyme Q10 (CoQ10) in patients with statin

myopathy. The evidence at present is contradictory. In a small study of patients with statin myopathy randomized to CoQ10 or vitamin E there was an improvement in muscle symptoms in those treated with CoQ10, but not vitamin E (Ahmed et al., 2009). A divergent result was reported by Young et al. (2007); in this study patients were either pretreated with CoQ10 or a placebo and then had an uptitration of simvastatin from 10 to 40 mg over 12 weeks. At the end of the study, despite a significant rise in serum CoQ10 concentrations, there was no difference in muscle symptoms or in the dose of simvastatin tolerated between the two groups. In patients who are intolerant of statin therapy the switch to another class of medication may be required; the options are ezetimibe, a fibrate, nicotinic acid, bile acid binding resins or fish oils. Ezetimibe as monotherapy can be expected to lead to a fall in LDL-C of approximately 15-20%, with a much lower risk of statin myopathy (Knopp et al., 2003). If muscle symptoms arise on ezetimibe therapy, then thrice-weekly dosing may be tolerated. In a small retrospective study of 94 statin-intolerant patients, a thrice-weekly regimen resulted in a fall in LDL-C of 17-20% and was tolerated by 85% of patients (Maccubin et al., 2009). Fibrates are effective in reducing cardiovascular disease and are an attractive option for those who are statin and ezetimibe intolerant. Fenofibrate has largely replaced gemfibrozil owing to a lower risk of drug interactions. Recent data indicate that fenofibrate is well tolerated and particularly effective in reducing microvascular complications in diabetic patients. Nicotinic acid (vitamin B3) is extremely effective in reducing LDL-C and triglycerides and raising high-density lipoprotein cholesterol. Unfortunately, nicotinic acid is associated with significant adverse effects, most notably skin flushing, and itch mediated through prostaglandin D2 release; these side effects are moderately to significantly intolerable in 20% of patients (Maccubin et al., 2009). Newer formulations such as niaspan (a slow release preparation) and niacin combined with laropiprant (a prostaglandin D2 inhibitor) are better tolerated and are effective in improving the lipid profile. Finally, bile acid sequestrants are also effective in improving reducing cholesterol levels; however, older preparations such as cholestyramine have significant gastrointestinal side effects and interfere with medication absorption. Newer preparations such as colesevelam have improved tolerability (<http://www.bma.org.uk/ap.nsf/>). Finally, recommending 'nutriceuticals', such as plant sterols, policosanol and Chinese red rice (containing naturally occurring lovastatin) may be useful in sparing the use, or in increasing the dose, of a statin. These supplements can reduce LDL-C by up to 10% (Becker et al., 2009). For patients with LDL-C > 4 mmol/L (160 mg/dl) who cannot tolerate or are refractory to cholesterol-lowering drugs and exhibit progressive coronary artery disease, consideration should be given to LDL-apheresis

(<http://www.bma.org.uk/ap.nsf/>)(Table 8).

DISCUSSION

This systemic meta-analysis demonstrates that there is association between non-adherence to statin therapy and its impact on cardiac events. For patients with good adherence to statin therapy, the risk of cardiovascular events is significantly reduced than that of patients with poor adherence. Non-adherence to statin therapy is a frequently occurring problem in Cardiology. Non-adherence is widely observed in clinical settings, suggesting discrepancy in patient adherence with statin therapy between controlled clinical trials and routine practice. Adherence to statin therapy in clinical trials tends to be much higher than every day practice. Non-adherence to cardiovascular medications is ubiquitous. It is chiefly seen in primary prevention settings, but also paradoxically so amongst patients with diabetes and stroke (Bates et al., 2009). This may be due to, in primary prevention setting patients may be less willing to receive statin since they perceive their vascular risk to be low (Search, 2008).

The reason for non-adherence to statin therapy relate inter alia to patient, physician, and fiscal factors. Several factors are shown to be predictive of poor adherence, such as age, race, education level, house hold income, family support, cigarette smoking, patients' beliefs, comorbidities, number of concurrent medications and drug side effects. Previous studies revealed that patients aged ≥ 60 years were better adherents, whereas those under 45 or over 75 years old showed significantly lower adherence rates (Benner et al., 2002). Elderly patients are a group who may be at particular risk of statin non-adherence. There are several reasons for this. These include polypharmacy, susceptibility to drug side effects, cognitive dysfunction, physical disability (poor eyesight, arthritis) and depression. The younger are also at risk for non-adherence because they do not take responsibility for their own health (Wilson et al., 2007). The number of close family members was also significantly associated the amount of house hold care received by the elderly family members. Higher levels of adherence were reported among Caucasians compared with African-origin groups (Benner et al., 2002). The reasons that the black is less adherent than the white is may be: chronic stress due to direct and indirect effect of racism, culture of diet and accessibility to care because of economy (Shear et al., 1992). Higher education and house income, cohesive family, positive attitude towards healthy living and patient-health care provider relationship may be correlated with increased adherence levels (Benner et al., 2002). Heavy smoking, complex regimens and presence of intolerable side effects may deter patients from adhering to the treatment (Colivicchi et al., 2007).

Adverse effects of statins experienced by patients are also an important cause of non-adherence to medication. There is a core of patients who are non-adherent to statins because of clinical side effects, most frequently myopathic and neuromuscular symptoms (Golomb and Evans, 2008). Non-adherence to statin has a significant effect on cardiovascular outcomes. A study by Bouchard et al (2007) indicted that adherence to statins that exceeds 90% is associated with a significant reduction in nonfatal CAD events. The 4S study (Strandberg et al., 1994) demonstrated that important reductions in coronary event-related morbidity and mortality in patients with known CHD.

A recent evidence has demonstrated that patients' financial incentives (that is, copayments, coinsurance) also affect statin adherence. As statin cost-sharing levels increase, adherence to statin falls. Reduction in drug costs, increase in government subsidies and reduction in patient copayments are other adjunctive approaches that will assuage non-adherence. Well-designed research studies into the most cost-efficient and strategies for improving adherence should be given priority for funding. Evidence-based government initiatives can have direct and indirect effects that improve patient adherence to cardiovascular drugs, including statins; this may be particularly relevant to primary prevention where adherence is a major problem (McGovern et al., 2008).

Psychological issues may bear on a patients' adherence behavior. An individual's personality and adherence self-efficacy are also important predictors of non-adherence and physicians who improve their levels of communication and emotional intelligence are more likely to understand and collaborate with their patients and reduce the incidence of non-adherence. Use of evidence-based medicine, clear communication with patients and a concerted commitment to regular follow-up of patients are likely solutions. Attention to the physician-patient working alliance and patient health beliefs will also promote adherence behavior (Bates et al., 2009). CoQ10 supplementation, identification of recognized drug interactions and use of low-dose regimens of lipophilic statins can improve patient acceptability. That said, there will be a small proportion of patients that remain intolerant of all statins and, depending on the lipoprotein profile, will require alternative drug therapy, including ezetimibe, fenofibrate or fish oils (Bates et al., 2009). The present review has limitations: relevant studies may have been missed or in correctly categorized, because one person selected the studies and extracted data. Another limitation is that the absence of an ideal method to measure adherence, a wide variety of measurement and definitions for adherence.

CONCLUSION AND RECOMMENDATIONS

In this meta-analysis non-adherence to statin therapy

proved to be a significant problem for preventive cardiology. It is associated with increased risk of cardiac morbidity and mortality. Non-adherence of statin medication is not solely a patient problem but is impacted by both care providers and health care system. Most studies investigating the relationship between adherence and clinical outcomes found that non-adherence had a negative effect on outcome; suggesting that the management of CVD may be improved by improving patient adherence to statins medication.

Further research into the problem of non-adherence with statin medication is necessary to increase the number of published studies in this area and to increase awareness of the problem. By increasing awareness, it may be possible to improve patient adherence. The availability of different targeted interventions, including behavioral training and regular follow up with health care system designed specifically to improve patient adherence, and hence to improve clinical outcomes. Beside this, getting patients to take their medications as prescribed is a worthy goal for patients to derive the maximal benefit of prescribed therapy.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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ABBREVIATIONS

ACE, Angiotensin converting Enzyme; **AFCAPS/texCAPS**, Air Force/Texas Coronary Atherosclerosis Prevention Study; **ASTEROID**, A Study to Evaluate the Effect of Rosuvastatin on Intravascular Ultrasound-Derived Coronary Atheroma Burden; **ATP III**, Adult Treatment Panel III; **LYS**, cost per life year saved; **CABGE**, Coronary Artery Graft Bypass surgery; **CARE**, Cholesterol and Recurrent Events; **CHAMP**, Cardiovascular Hospitalization Atherosclerosis Management Program; **CHD**, Coronary Heart Disease; **CURVES**, Comparative dose efficacy study of atorvastatin versus simvastatin; **EXCEL**, Expanded Clinical Evaluation of Lovastatin; **GRACE**, Gender, Race and Clinical Experience; **HMG-COA**, Hydroxy methyl glutaryl coenzyme A; **HMO**, Health Maintenance organizations; **HPS**, Heart Protection Study; **IDEAL**, Incremental Decrease in End Points through Aggressive Lipid Lower; **JUPITER**, Justification for the Use of Statins

in Primary Prevention: An Intervention Trial Evaluating Rosuvastatin trial; **LDL-C**, Low density lipoprotein cholesterol; **LIPID**, Long-Term Intervention with Pravastatin in Ischaemic Disease; **MACE**, Major adverse cardiovascular events; **NCEPG**, National Cholesterol Education program guidelines; **SPARCL**, Stroke Prevention by Aggressive Reduction in Cholesterol Levels; **TIA**, Transient Ischemic Attack; **VLDL-C**, Very low-density lipoprotein cholesterol; **BMQ**, Beliefs about Medicines Questionnaire; **WOSCOPS**, West of Scotland Coronary Prevention Study; **4S study**, Scandinavian Simvastatin Survival Study; **WAI**, Working Alliance Inventory.

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Full Length Research Paper

Hepatoprotective and antioxidant activities of *Pterocarpus santalinoides* methanol leaf extract

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This study evaluated the hepatoprotective and antioxidant activities (AA) of *Pterocarpus santalinoides* methanol leaf extract (PSMLE) on carbon tetrachloride (CCl₄)-induced hepatotoxicity in albino rats. Thirty male albino rats randomly assigned into 6 groups (A – F) of 5 rats each were used for the *in vivo* study. Hepatotoxicity was induced in groups A – E using CCl₄. Group A served as negative control. Groups B, C and D were treated with 50, 250 and 500 mg/kg PSMLE, respectively. Group E was treated with 100 mg/kg Silymarin, while Group F served as normal control. Treatment was given orally twice daily for 15 days, after which markers of hepatotoxicity and oxidative stress were evaluated. The *in vitro* AA of PSMLE was also evaluated using 1, 1-diphenyl 2-picryl hydrazyl. Results showed that treatment with PSMLE at 250 and 500 mg/kg led to significantly ($p < 0.05$) lower serum alanine aminotransferase and malondialdehyde, significantly ($p < 0.05$) higher superoxide dismutase and glutathione peroxidase levels, while 250 mg/kg dose further led to significantly ($p < 0.05$) lower serum aspartate aminotransferase and serum total bilirubin levels, and significantly ($p < 0.05$) higher serum total protein and serum globulin levels. 500 mg/kg dose treatment additionally led to significantly ($p < 0.05$) lower serum total cholesterol. Treatment at all doses led to significantly ($p < 0.05$) lower liver weight and relative liver weights and significantly ($p < 0.05$) higher catalase and total glutathione levels. The PSMLE exhibited significantly ($p < 0.05$) higher AA at concentrations ≥ 50 $\mu\text{g/ml}$ *in vitro*. It was concluded that PSMLE was hepatoprotective and possesses significant antioxidant activity *in vivo* and *in vitro*.

Key words: Hepatotoxicity, oxidative stress, antioxidants, *Pterocarpus santalinoides* leaf extract, carbon tetrachloride.

INTRODUCTION

The liver is a vital organ for metabolism, excretion, clearance and transformation of chemicals in the body (Singh et al., 2011). It is responsible for the detoxification of drugs and xenobiotics; thus, it is constantly and

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variedly exposed to xenobiotics which may induce liver damage (Saukkonen et al., 2006). Most absorbed toxins and toxicants will first pass through liver, and the possible response elicited may range from inflammation to degeneration and/or neoplasia of the hepatocytes (Schiff and Schiff, 1987). Hepatotoxicity is a major health problem, and the manifestations vary from asymptomatic elevation of liver enzymes to fulminant liver failure (Saukkonen et al., 2006). Toxic liver damage is commonly oxidative stress mediated, and constitutes a large proportion of liver disorders/diseases; its occurrence has been steadily increasing over the years (Suk and Kim, 2012; Rehm et al., 2013; Nwokediuko et al., 2013).

Carbon tetrachloride (CCl_4) is a commonly used model chemical for the experimental induction of hepatotoxicity (Kim et al., 2010). It is metabolized to trichloromethyl (CCl_3) free radical which induces hepatotoxicity by causing peroxidative degradation in the adipose tissue, resulting in fatty infiltration of the hepatocytes (Boll et al., 2001). Following administration, CCl_4 is activated by cytochrome CYP_{2E1} and CYP_{2B1} to form CCl_3 radical which binds to cellular molecules such as nucleic acids, proteins and lipids, thereby impairing crucial cellular processes like lipid metabolism, with the potential outcome of fatty degeneration (Boll et al., 2001). The CCl_3 radical reacts with oxygen to form highly reactive species, the trichloromethylperoxy (CCl_3OO) radical, which initiates the chain reaction of lipid peroxidation culminating in destruction of polyunsaturated fatty acids (Boll et al., 2001). This causes alteration in permeability of the mitochondria, endoplasmic reticulum, and plasma membranes, resulting in the loss of cellular calcium, disruption of calcium homeostasis and damage/death of hepatocytes (Weber et al., 2003).

Oxidative stress is a state in which oxidation and oxidants exceed the antioxidant systems in the body leading to imbalance between the generation of reactive oxygen species (ROS) and the level of antioxidants in the biological system (Yoshikawa and Naito, 2002). It occurs when free radicals which are not neutralized by antioxidants go on to create more volatile free radicals and damage cell membranes, vessels, proteins, fats and DNA. Biological free radicals are highly unstable reactive molecules that have electrons available to react with various organ substrates such as DNA, proteins and lipids. Oxidative stress is known to be involved in the pathogenesis of a variety of diseases including atherosclerosis, hypertension, diabetes mellitus, ischemic diseases, liver diseases and malignancies (Yoshikawa and Naito, 2002), or may exacerbate their symptoms (Halliwell and Gutteridge, 1989; Valko et al., 2007).

Antioxidants are compounds that inhibit the oxidation of other compounds and prevent chemical damage caused by free radicals (Sies, 1997). Oxidation reactions in living organisms produce free radicals which can initiate chain reactions that may cause damage or death to cells.

Antioxidants terminate these chain reactions by removing free radical intermediates and inhibiting other oxidation reactions (Sies, 1997; Valko et al., 2007). Insufficient levels of anti-oxidants or inhibition of the antioxidant enzymes in living organisms cause oxidative stress which may lead to injury and/or death of cells (Davies, 1995; Valko et al., 2007). Many of the natural antioxidants such as tannins, flavonoids and glycosides are very important in the prevention of diseases associated with oxidative stress (Yi-Fang et al., 2002; Aruoma, 2003).

Some plants such as *Cussona barteri* (leaves), *Lannea vilutina* (leaves), *Sacoglottis gabonensis* (stem bark), *Trichilia roka* (roots), *Tinospora cordifolia* (whole plant), *Piptadeniastrum africanum* (stem bark) and *Gongronema latifolium* (leaf) amongst others, have been reported to be rich sources of natural antioxidants that can protect against oxidative stress and thus play important role in the chemoprevention of diseases that have their etiology and pathophysiology in ROS (Ames et al., 1993; Atawodi, 2005; Karamalakova et al., 2018; Dlamini et al., 2019). There has been an increase in interest in the therapeutic potential of plants as antioxidants that may reduce free radical-induced tissue injury (Schuler, 1990; Karamalakova et al., 2018). A number of plants such as *Ipomoea batatas* (leaves), *Allium cepa* (leaves), *Cnestus ferruginea* (leaves stem and roots), *Splenacentrum jollyanum* (leaves and roots) and *Voacanga africana* (leaves) had been investigated in the search for novel antioxidants (Chu, 2000; Mantle et al., 2000; Koleva et al., 2002; Oke and Hamburger, 2002), while a lot more are still under investigation.

Pterocarpus santalinoides DC is an indigenous Nigerian plant in the family *Papilionaceae* (Keay, 1989). It is commonly known as “red sandal wood” in English language and “nturukpa” in Igbo language (Adetunji, 2007; Anowi et al., 2012). Leaves of *P. santalinoides* are used traditionally as vegetable for making soup, and also in folk medicine for the treatment of various ailments including heart and liver diseases (Adesina, 1982; Okwu and Ekeke, 2003). Leaves of *P. santalinoides* contain considerable amounts of tannins, flavonoids and glycosides which are known natural antioxidants (Anowi et al., 2012; Eze et al., 2012; Ihedioha et al., 2017; 2018). Previous studies by Ihedioha et al. (2017) suggested that the hepatoprotective properties of methanol leaf extract of *P. santalinoides* in acetaminophen-induced hepatotoxicity may be attributed to its antioxidant phytochemical composition. Also, reports from earlier studies on the lipid lowering effects of aqueous leaf infusion of *P. santalinoides* in guinea pigs showed that it may be related to its antioxidant properties (Ihedioha et al., 2018). Based on the various traditional medicinal uses of *P. santalinoides* especially in the treatment of diseases in which oxidative stress is known to play critical role and the results of these earlier cited studies, the purpose of the present study was to evaluate the

hepatoprotective and antioxidant activities of methanol leaf extract of *Pterocarpus santalinoides* on CCl₄-induced sub-acute hepatotoxicity *in vivo* in albino rats, and *in vitro* using the DPPH assay method.

MATERIALS AND METHODS

Plant collection, identification and extract preparation

Fresh leaves of *Pterocarpus santalinoides* (Figure 1) used for the study were collected from Nsukka Local Government Area of Enugu State in November 2017. The plant was identified and authenticated by a plant taxonomist (Mr. A.O. Ozioko) at the Department of Plant Science and Biotechnology, University of Nigeria, Nsukka, a voucher specimen [UNH (University of Nigeria Herbarium) No. 02] was deposited at the University of Nigeria, Nsukka, herbarium. The leaves were dried under shade and pulverized. Five hundred grammes of the pulverized leaves were extracted with 80% methanol using the cold maceration extraction technique. The resulting extract was filtered with Whatman size 1 filter paper, concentrated to dryness with a Rotary Evaporator (Buchi, Switzerland), and referred to as *Pterocarpus santalinoides* methanol leaf extract (PSMLE).

Experimental animals

Thirty adult male albino rats (*Rattus norvegicus*) of 12 weeks of age, weighing between 200 and 250 g, were obtained from the Laboratory Animal House of the Department of Veterinary Physiology and Pharmacology, University of Nigeria, Nsukka, and used for the *in vivo* hepatotoxicity study. Fifteen adult female rats of 12 weeks of age weighing between 172- 190 g were also obtained from the same source and used for the acute toxicity study. The albino rats were housed in stainless steel cages in a fly proof Animal House at room temperature between 23 -29°C, and allowed 2 weeks to acclimatize before the commencement of the study. They were fed commercial rat pellets (Grand Cereals Nig. Ltd, Jos, Nigeria), composed of 13% crude protein, 8% fat, 15% crude fibre, 0.9% calcium, 0.35% phosphorus and 2600 Kcal/kg metabolizable energy, and clean drinking water *ad libitum*. They were cared for and handled humanely all through the study. Stipulated guidelines governing the use of animals for laboratory experiments were strictly adhered to (Zimmermann, 1983; Ward and Elsea, 1997). The protocol for the laboratory animal study was approved by the Faculty of Veterinary Medicine Institutional Animal Care and Use Committee, University of Nigeria, Nsukka, (Approval No: FVM-UNN-IACUC/2018/0814).

Acute toxicity study

The acute toxicity and median lethal dose (LD₅₀) of PSMLE were determined in albino rats following the OECD Acute Toxic Class method (OECD, 2001). Fifteen adult female albino rats, randomly assigned into 5 groups of 3 rats each (Groups 1, 2, 3, 4 and 5) were used for testing at 0, 50, 300, 2000 and 5000 mg/kg, respectively. The albino rats were fasted for 12 hours before the test commenced, but water was made available to them *ad libitum*. The extracts were each dissolved in 1 ml of distilled water and administered orally with a gastric tube. The rats were observed for 14 days for any sign of toxicity or mortality. Their body weights were

measured at intervals, and at the end of the 14 days of observation, they were humanely sacrificed and the weights of their vital organs (liver, kidney, spleen and heart) were measured (OECD, 2001).

Phytochemical analysis of PSMLE

Phytochemical analysis was done to determine the phytochemical constituents of PSMLE, following standard procedures as described by Trease and Evans (1996), Harborne (1998). One gramme (1 g) of PSMLE was dissolved in 100 ml of distilled water in a beaker. The solution was filtered with Whatmann no. 1 filter paper to obtain a clear filtrate which was used to test for the presence of tannins, flavonoids, alkaloids, saponins, phenols, carbohydrates, glycosides, starch, polyuronides, sterols and terpenes. High level presence of each phytochemical was scored +++, moderate levels were scored ++, low levels were scored + (Trease and Evans, 1996; Harborne, 1998).

Evaluation of the effects of PSMLE on blood levels of markers of hepatotoxicity, oxidative stress and antioxidant marker levels in albino rats given sub-acute toxic doses of CCl₄

The thirty male albino rats used for the hepatotoxicity and oxidative stress study were randomly assigned to 6 groups A – F of five rats each. Hepatotoxicity and oxidative stress were induced in rats in groups A – E using the CCl₄ model (Robin et al., 2012; Singh et al., 2012). A mixture of 1 ml/kg CCl₄ in equal volume of olive oil (50% v/v) was injected intraperitoneally to groups A – E at 3 day intervals (days 0, 3, 6, 9 and 12) for 12 days. Group A was treated with 10 ml/kg distilled water as placebo and served as negative (untreated) control, Groups B, C and D were treated with 50, 250 and 500 mg/kg PSMLE, respectively. Group E was treated with 100 mg/kg Silymarin (a known hepatoprotective and antioxidant drug) as positive control, while Group F was also given 10 ml/kg distilled water as placebo and served as normal control (not given CCl₄). Treatment started 24 h post-initial CCl₄ administration and was done twice daily for 15 days. On day 15 post-initial CCl₄ administration, two milliliters of blood sample was collected from each rat and used immediately for evaluation of blood levels of enzyme markers of liver damage, oxidative stress and antioxidant markers, following standard procedures. Blood sample collection was done using the orbital technique (Bolliger and Everds, 2010). A portion of the blood (1.5 ml) was dispensed into a glass test tube and allowed to stand at room temperature for 45 min to clot; then centrifuged at 3000 revolutions per minute for ten minutes using a table centrifuge (Jenalab Medical, England), after which the serum was harvested and assayed immediately. The remaining 0.5 ml was dispensed into a heparinized sample bottle for use in the determination of glutathione peroxidase activity.

Evaluation of effects of PSMLE on blood levels of markers of hepatotoxicity

Serum alanine aminotransferase (ALT) and serum aspartate aminotransferase (AST) activities were evaluated following the Reitman and Frankel method (Colville, 2002), using the Quimica Clinica Applicada (QCA) serum ALT and AST test kits. The ALT in the serum sample and standards catalyzed the reaction of L-alanine and alpha-ketoglutaric acid to form pyruvic acid and L-glutamic acid, while the AST catalyzed the reaction of L-aspartic acid with alpha- ketoglutaric acid to form oxaloacetic acid and L-glutamic acid. These ketonic acids reacted with 2-4, dinitrophenyl hydrazine to form corresponding colored hydrazones, the optical



Figure 1. Leaves of *Pterocarpus santalinoides*.

density of which was measured and ALT/AST quantified at 505 nm wavelengths, using the Diatek[®] semi-automated blood biochemistry analyzer (Diatek Instruments, Wuxi, China).

The serum alkaline phosphatase (ALP) activity was quantified using the QCA alkaline phosphatase test kit, based on the phenolphthalein monophosphate method (Colville, 2002). The alkaline phosphatase in the serum and a standard (containing 30 IU/L alkaline phosphatase) hydrolyzed a colorless substrate of phenolphthalein monophosphate and gave rise to phosphoric acid and phenolphthalein which at alkaline pH turned into pink color. The optical density was measured and the alkaline phosphatase activity was quantified at 546 nm wavelength, using the semi-automated biochemistry analyzer (Diatek Instruments, Wuxi, China).

Serum total protein levels were determined using the QCA total protein test kit which was based on the direct Biuret method

(Lubran, 1978; Johnson, 2008). A reaction of the proteins in the serum samples and a standard (containing 5 g/dl of proteins), with copper ions in the Biuret reagent in an alkaline medium, resulted in the formation of a stable colored complex. The optical density of the colored complex was measured at 546 nm wavelength and quantified using the Diatek[®] semi-automated blood biochemistry analyzer (Diatek Instruments, Wuxi, China).

Assay of serum albumin was done using the QCA albumin test kit based on the bromocresol green method (Doumas and Peters, 1997; Johnson, 2008). This involved the reaction of the albumin in the serum samples and standard (containing 5 g/dl of albumin) with bromocresol reagent at acid pH to form a colored complex. The optical density of the colored complex was measured at 630 nm wavelength and the serum albumin was quantified using the Diatek[®] semi-automated blood biochemistry analyzer (Diatek

Instruments, Wuxi, China). The globulin levels were calculated by subtracting the serum albumin levels from the total protein levels (Johnson, 2008).

The serum total cholesterol levels were determined using the QCA total cholesterol test kit, which is based on the enzymatic colorimetric method (Allain et al., 1974; Rifai et al., 2008). Total cholesterol in the serum samples and standard (containing 200 mg/dl of cholesterol) was enzymatically hydrolyzed by cholesterol esterase and further oxidized by cholesterol oxidase contained in the QCA total cholesterol working reagent. The reactions resulted to formation of a colored quinonic derivative. The optical density of the colored quinonic solution was measured at 505 nm wavelength and the total cholesterol was quantified using the Diatek[®] semi-automated blood biochemistry analyzer (Diatek Instruments, Wuxi, China).

The total bilirubin levels in the serum samples was assayed using the Randox[®] bilirubin test kit (Randox Laboratories Ltd, County Antrim, United Kingdom), which is based on the Jendrassik and Grof method (Doumas et al., 1973; Higgins et al., 2008). The serum samples were reacted with diazotized sulfanilic acid in the presence of caffeine to produce an azopigment. Their optical densities were measured at 578 nm and quantified a Chem5V3[®] Semi-automated Clinical Chemistry Analyzer, (Erba Diagnostics, Mannheim GmbH, Mannheim, Germany).

Evaluation of effects of PSMLE on oxidative stress and antioxidant markers

The serum malondialdehyde (MDA) levels of the rats were determined following the modified thiobarbituric acid method (Draper and Hadley, 1990). The serum was first mixed with trichloroacetic acid (TCA) and centrifuged to obtain the protein-free supernatant, to which was added 1% thiobarbituric acid and incubated for one hour at 95°C. The free MDA present in the supernatant reacted with thiobarbituric acid (TBA) to generate an MDA-TBA adduct, which was quantified colorimetrically at 532 nm wavelength using a SpectrumLab[®] spectrophotometer (HME Global Medical, England).

The serum catalase (CAT) activity was determined by the visible light method (Weydert and Cullen, 2010), using ElabScience catalase assay kit (ElabScience Biotechnology Co., Ltd., South Africa). In this determination, the decomposition of H₂O₂ by catalase in the plasma sample was quickly stopped by ammonium molybdate, and the rest of H₂O₂ reacted with the ammonium molybdate to generate a yellowish complex. The absorbance of the yellowish complex was measured at the wavelength of 405 nm using a Chem5V3[®] Semi-automated Clinical Chemistry Analyzer, (Erba Diagnostics, Mannheim GmbH, Mannheim, Germany) and the catalase activity was calculated from the absorbance.

The superoxide dismutase (SOD) activity was determined on serum, using the ElabScience SOD Assay kit (ElabScience Biotechnology Company Ltd., South Africa), which was based on the hydroxylamine method that adopted xanthine oxidase to measure SOD activity (Weydert and Cullen, 2010) with a Chem5V3[®] Semi-automated Clinical Chemistry Analyzer, (Erba Diagnostics, Mannheim GmbH, Mannheim, Germany), set at 550 nm wavelength.

The glutathione peroxidase (GPx) activity was determined on heparinized whole blood using a Fortress Diagnostics GPx test kit (Fortress Diagnostics Ltd, Antrim, UK.), based on the method described by Weydert and Cullen (2010). In this determination, the GPx in blood catalysed the oxidation of glutathione (GSH) by cumene hydroperoxide. The oxidized glutathione was converted to the reduced form in the presence of glutathione reductase and NADPH, and the NADPH was oxidized to NADP⁺ simultaneously.

The absorbance of the solution at 340 nm wavelength was measured using a Chem5V3[®] Semi-automated Clinical Chemistry Analyzer, (Erba Diagnostics, Mannheim GmbH, Mannheim Germany) and the GPx activity concentration was calculated from the absorbance.

The serum total glutathione (GSH) was determined using RayBio[®] Glutathione Colorimetric Detection Kit (RayBiotech Inc., Georgia, USA), which was based on the modified enzyme recycling system by 5, 5-dithiobis-(2-nitrobenzoic acid) (DNTB) and glutathione reductase (Tippel and Rogers, 2012). The DNTB and glutathione reacted to generate 2-nitro-5-thiobenzoic acid which has a yellow color. The glutathione concentration was determined by measuring the absorbance of the generated yellow solution at 412 nm using Diatek[®] DR-3508G Microplate (ELISA) Reader, (Wuxi Hiwell Diatek Instruments Co. Ltd., China). The total glutathione concentration in the plasma was obtained from the standard total glutathione calibration curve.

Measurement of the liver weight and the calculation of the relative liver weight (liver weight percentage of body weight)

After blood sample collection, the rats were euthanized by intraperitoneal injection of 250 mg/kg Thiopentone sodium and confirmatory exsanguination (AVMA, 2013). The liver of each rat was carefully eviscerated and weighed, and the relative liver weight (liver weight percentage of the body weight) was calculated.

Evaluation of the *in vitro* antioxidant activity (AA) of PSMLE using (DPPH) assay method

The *in vitro* anti-oxidant activity of PSMLE was analyzed following the 1, 1-diphenyl 2-picryl hydrazyl (DPPH) assay method (Mensor et al., 2001). Two milliliters of PSMLE in distilled water at concentrations of 10, 50, 100, 200 and 400 µg/ml were each mixed with 1 ml of 0.5 mM DPPH (in methanol) in a cuvette. The DPPH reagent formed yellow coloured complexes with the free hydroxyl group present in the crude extract. The absorbance of the coloured complex was read at 517 nm wavelength after 30 minutes of incubation in the dark at room temperature, with the aid of a spectrophotometer (HME Global Medical, England). The tests were done in triplicates. A mixture of 1 ml of methanol and 2 ml of PSMLE served as blank, while 1 ml of 0.5 mM DPPH solution and 2 ml of methanol served as negative control. Ascorbic acid was used as the reference standard. The percentage AA was calculated as follows:

$$\% \text{ AA} = 100 - \left[\frac{\text{Absorbance of sample} - \text{Absorbance of blank}}{\text{Absorbance of control}} \times 100 \right]$$

(Mensor et al., 2001; Oke and Hamburger, 2002).

Data analysis

Data obtained from the *in vivo* experiment with rats, and the results of PSMLE and ascorbic acid across the different concentrations, were subjected to one way analysis of variance (ANOVA). Variant means were separated post-hoc using the least significant difference method. Significance was accepted at $p < 0.05$. The *in vitro* anti-oxidant activities of PSMLE at the varied concentrations were compared with that of ascorbic acid using the student's t-test. A summary of the results are presented as bar charts with standard deviation bars and as table of means with standard error.

Table 1. Mean \pm standard error of the body weights (g) and percentage change in body weights of albino rat groups* given graded acute oral doses of *Pterocarpus santalinoides* methanol leaf extract (PSMLE).

Rat groups	Body weights (g) of the rats				% change in body weights
	Day 0	Day 3	Day 7	Day 14	
Group 1	183.17 \pm 8.02	186.37 \pm 8.09	187.57 \pm 8.51	189.47 \pm 8.45	3.42 \pm 1.31
Group 2	181.23 \pm 10.96	182.57 \pm 10.63	184.20 \pm 10.34	187.73 \pm 8.92	3.83 \pm 1.30
Group 3	179.73 \pm 6.06	182.10 \pm 6.30	182.33 \pm 4.91	186.40 \pm 4.73	3.71 \pm 0.83
Group 4	182.36 \pm 7.84	184.03 \pm 6.93	184.88 \pm 8.47	189.41 \pm 7.52	3.85 \pm 1.29
Group 5	180.30 \pm 8.19	181.43 \pm 7.74	183.78 \pm 8.01	187.58 \pm 7.57	4.04 \pm 1.40

No significant differences ($p > 0.05$) in the body weight and percentage change in body weight of the rats groups. * Groups: Group 1 - 0 mg/kg PSMLE (untreated); Group 2 - 50 mg/kg PSMLE; Group 3 - 300 mg/kg PSMLE; Group 4 - 2000 mg/kg PSMLE; and Group 5 - 5000 mg/kg PSMLE.

Table 2. Mean \pm standard error of the organ weights (g) of albino rat groups* given graded acute oral doses of *Pterocarpus santalinoides* methanol leaf extract (PSMLE).

Rat groups	Organ weights (g)			
	Liver weight	Average kidney weight	Spleen weight	Heart weight
Group 1	6.85 \pm 0.34	0.61 \pm 0.02	0.72 \pm 0.05	0.63 \pm 0.05
Group 2	6.76 \pm 0.22	0.60 \pm 0.05	0.70 \pm 0.03	0.62 \pm 0.04
Group 3	6.69 \pm 0.23	0.58 \pm 0.01	0.69 \pm 0.05	0.65 \pm 0.03
Group 4	6.78 \pm 0.24	0.60 \pm 0.06	0.71 \pm 0.04	0.63 \pm 0.04
Group 5	6.74 \pm 0.21	0.59 \pm 0.02	0.67 \pm 0.05	0.62 \pm 0.05

No significant differences ($p > 0.05$) in the organ weights of the rat groups. * Groups: Group 1 - 0 mg/kg PSMLE (untreated); Group 2 - 50 mg/kg PSMLE; Group 3 - 300 mg/kg PSMLE; Group 4 - 2000 mg/kg PSMLE; and Group 5 - 5000 mg/kg PSMLE.

RESULTS

Acute toxicity

There was no sign of toxicity or abnormality and no mortality in all the rat groups given the varied acute doses (0, 50, 300, 200 and 5000 mg/kg) of PSMLE all through the 14 day observation/monitoring period. All the rats which were given the varied acute doses were normal, with no sign of changes in the behavioral pattern, mucous membrane, skin and eyes. There were no significant differences ($p > 0.05$) in the body weights and the percentage change in body weights of the rat groups treated with the varied acute doses of PSMLE when compared to the untreated control (Table 1). There were also no significant differences ($p > 0.05$) between the groups given the acute doses of PSMLE and the untreated in their organ weights and relative organ weights (organ weight percentages of body weights) (Tables 2 and 3).

Phytochemical analysis

Phytochemical analysis revealed the presence of high

(+++ levels of tannins, glycosides, carbohydrates, saponins, phenols, sterols and terpenes; moderate (++) levels of flavonoids and low (+) level of alkaloid (Table 4).

Effects of PSMLE on blood levels of markers of hepatotoxicity

Treatment with 250 and 500 mg/kg PSMLE (Groups C and D) led to significantly ($p < 0.05$) lower serum ALT activity (Table 5). The 250 mg/kg PSMLE dose also led to significantly ($p < 0.05$) lower serum AST activity when compared to the negative control (Group A), and compared favorably with that of Group E (100 mg/kg silymarin) and Group F (normal control) rats (Table 5). There was no significant ($p > 0.05$) differences in the ALP activity between all the PSMLE-treated groups (Groups B, C and D) and the negative control (Group A), though the recorded lowering of the ALP activity in the treated groups occurred in a dose dependent manner (Table 5). The ALP activity of the normal control (Group F) was significantly ($p < 0.05$) lower than that of the negative control and all the PSMLE-treated groups (Table 5). Treatment with PSMLE at 250 mg/kg (Group C) led to

Table 3. Mean \pm standard error of the organ weight percentages (relative organ weights) of albino rats groups* given graded acute oral doses of *P. santalinoides* methanol leaf extract (PSMLE).

Rat groups	Relative organ weights (%)			
	Liver weight %	Kidney weight %	Spleen weight %	Heart weight %
Group 1	3.54 \pm 0.03	0.32 \pm 0.01	0.37 \pm 0.02	0.33 \pm 0.01
Group 2	3.61 \pm 0.05	0.32 \pm 0.01	0.38 \pm 0.02	0.32 \pm 0.01
Group 3	3.59 \pm 0.05	0.31 \pm 0.02	0.37 \pm 0.02	0.35 \pm 0.02
Group 4	3.60 \pm 0.04	0.32 \pm 0.01	0.38 \pm 0.02	0.33 \pm 0.02
Group 5	3.60 \pm 0.04	0.32 \pm 0.01	0.36 \pm 0.02	0.33 \pm 0.02

No significant differences ($p > 0.05$) in the relative organ weights of the rats groups. * Groups: Group 1 - 0 mg/kg PSMLE (untreated); Group 2 - 50 mg/kg PSMLE; Group 3 - 300 mg/kg PSMLE; Group 4 - 2000 mg/kg PSMLE; and Group 5 - 5000 mg/kg PSMLE.

Table 4. Phytochemical constituents of *P. santalinoides* methanol leaf extract (PSMLE).

Phytochemical constituents	Semi-quantitative composition
Tannins	+++
Terpenes and sterols	+++
Alkaloids	+
Flavonoids	++
Saponins	+++
Carbohydrates	+++
Glycosides	+++
Phenol	+++

+++ = High levels present; ++ = Moderate levels present; + = Low levels present.

Table 5. Effects of oral administration of graded doses of PSMLE on serum enzyme activities of albino rats given sub-acute toxic doses of CCl_4

Group	Mean \pm standard error (U/L)		
	ALT	AST	ALP
Group A	102.4 \pm 4.79 ^a	121.03 \pm 8.03 ^a	384.46 \pm 60.81 ^a
Group B	98.78 \pm 6.83 ^a	120.82 \pm 7.41 ^a	391.01 \pm 46.11 ^a
Group C	48.36 \pm 7.02 ^b	77.28 \pm 7.96 ^b	358.42 \pm 10.08 ^a
Group D	79.11 \pm 6.08 ^c	106.92 \pm 5.16 ^a	338.41 \pm 18.93 ^a
Group E	46.96 \pm 7.48 ^b	75.03 \pm 6.74 ^b	279.97 \pm 22.86 ^{ab}
Group F	30.83 \pm 4.61 ^b	63.01 \pm 6.42 ^b	104.11 \pm 21.04 ^b

^{a, b, c} Different alphabetical superscripts in a column indicate significant ($p < 0.05$) differences between the groups.

*Groups: Group A – CCl_4 + 10 ml/kg distilled water (negative control); Group B – CCl_4 + 50 mg/kg PSMLE; Group C – CCl_4 + 250 mg/kg PSMLE; Group D – CCl_4 + 500 mg/kg PSMLE; Group E – CCl_4 + 100 mg/kg silymarin; Group F – No CCl_4 and no treatment.

significantly ($p < 0.05$) higher serum total protein levels when compared to the negative control (Group A) (Figure 2). Treatment with PSMLE at all the doses used in the study had no significant ($p > 0.05$) effect on the serum albumin levels (Figure 2). There was significantly ($p <$

0.05) higher serum globulin level in albino rats treated with 250 mg/kg PSMLE (Group C) when compared to the negative control (Group A). There was no significant ($p > 0.05$) differences in serum globulin levels between 250 mg/kg PSMLE-treated rats (Group C), 100 mg/kg

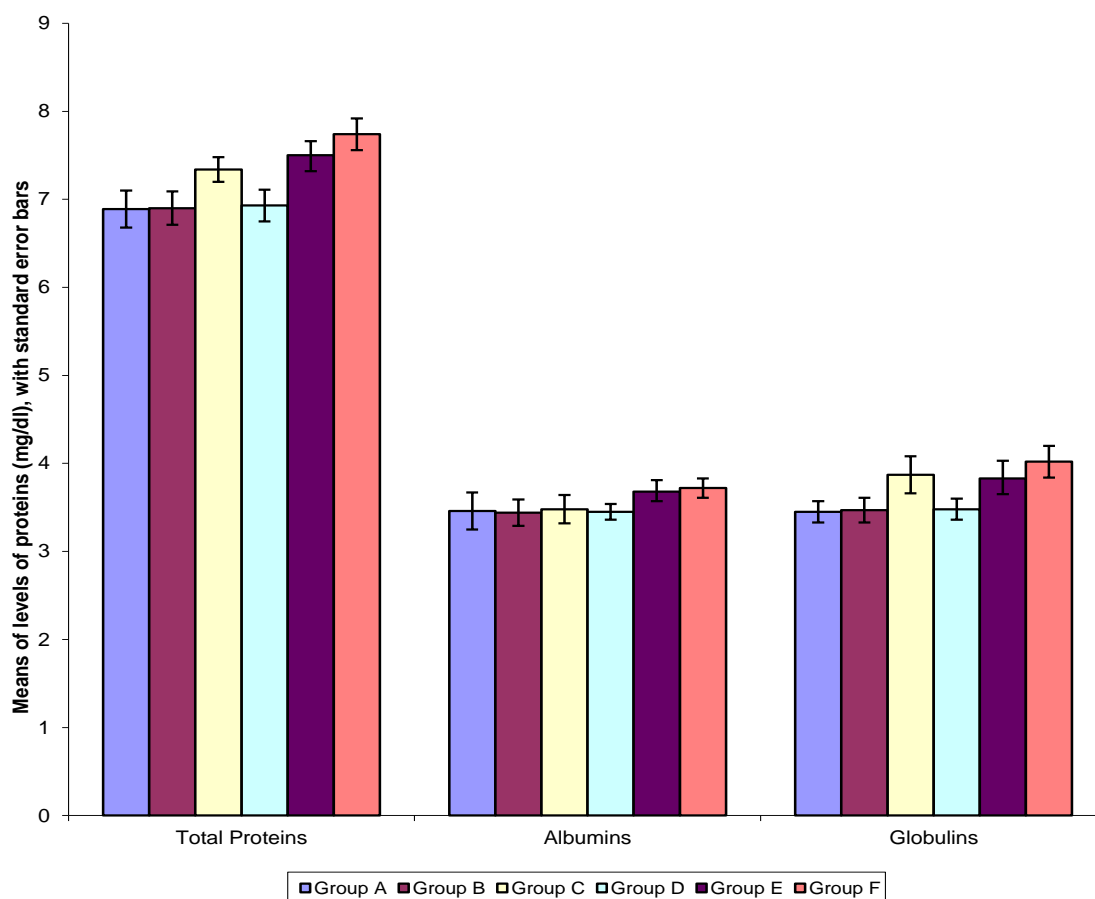


Figure 2. Effects of oral administration of graded doses of PSMLE on serum proteins of albino rats given sub-acute toxic doses of CCl_4 ; [Group A – CCl_4 + 10 ml/kg distilled water (negative control); Group B - CCl_4 + 50 mg/kg PSMLE; Group C - CCl_4 + 250 mg/kg PSMLE; Group D - CCl_4 + 500 mg/kg PSMLE; Group E - CCl_4 + 100 mg/kg Silymarin; Group F – No CCl_4 , No treatment (normal control)].

silymarin-treated (Group E) and the normal control (Group F) (Figure 2).

The serum total cholesterol level of Group D rats (500 mg/kg PSMLE) was significantly ($p < 0.05$) lower than that of the negative control (Group A), and there were no significant ($p > 0.05$) differences in serum total cholesterol levels between Group D and Groups B, C, E and F (Table 6). Treatment with 250 mg/kg PSMLE (Group C) and 100 mg/kg silymarin (Group E) significantly ($p < 0.05$) lowered serum total bilirubin levels when compared to the negative control (Group A) and other PSMLE-treated groups (Table 6). There was no significant ($p > 0.05$) difference in serum total bilirubin level between the rat groups treated with 250 mg/kg PSMLE, 100 mg/kg silymarin and the normal control (Table 6). The liver weight of the rat groups treated with 50, 250 and 500 mg/kg PSMLE and 100 mg/kg silymarin (Groups B, C, D and E) were significantly ($p < 0.05$) lower than that of the negative control (Group A), with Groups C, D and E being significantly ($p < 0.05$) lower than

Group B, and comparing favorably with the normal control (Group F) (Figure 3). Treatment with PSMLE at all doses used in the study (Groups B, C and D) and 100 mg/kg silymarin (Group E) led to significantly ($p < 0.05$) lower relative liver weights of the albino rats when compared to the negative control (Group A) (Figure 3).

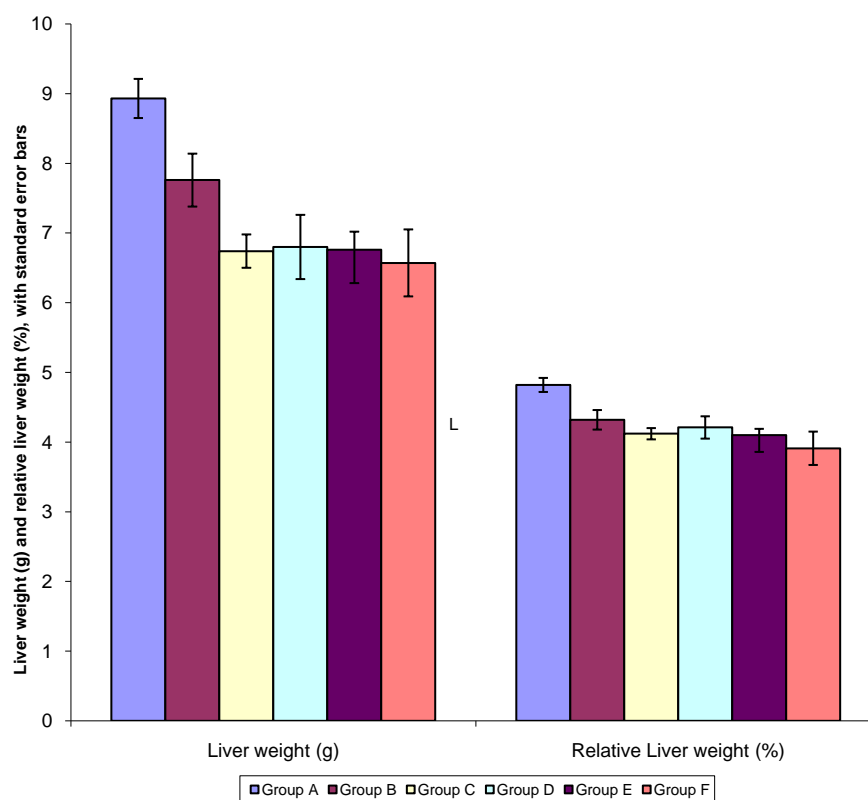
Effects of PSMLE on oxidative stress and antioxidant markers

Treatment with PSMLE caused a dose-dependent reduction in MDA levels across the groups, with the negative control (Group A) being significantly ($p < 0.05$) higher than 250 and 500 mg/kg PSMLE, 100 mg/kg silymarin and normal control (Groups C, D, E and F), but not significantly ($p > 0.05$) different from 50 mg/kg PSMLE (Group B) (Table 7). The CAT activity of Groups B, C, D and E was significantly ($p < 0.05$) higher than that of Group A rats, and there were no significant ($p > 0.05$)

Table 6. Effects of oral administration of graded doses of PSMLE on serum total cholesterol and serum total bilirubin levels of albino rats given sub-acute toxic doses of CCl₄.

Group	Mean \pm standard error (mg/dl)	
	Total cholesterol	Total bilirubin
Group A	81.23 \pm 8.04 ^a	0.70 \pm 0.06 ^a
Group B	63.48 \pm 10.84 ^{ab}	0.63 \pm 0.14 ^a
Group C	61.64 \pm 3.02 ^{ab}	0.36 \pm 0.07 ^b
Group D	56.48 \pm 2.42 ^b	0.66 \pm 0.03 ^a
Group E	64.19 \pm 5.76 ^{ab}	0.37 \pm 0.04 ^b
Group F	62.49 \pm 3.54 ^{ab}	0.38 \pm 0.03 ^b

^{a, b, c} Different alphabetical superscripts in a column indicate significant ($p < 0.05$) differences between the groups. *Groups: Group A – CCl₄ + 10 ml/kg distilled water (negative control); Group B – CCl₄ + 50 mg/kg PSMLE; Group C – CCl₄ + 250 mg/kg PSMLE; Group D – CCl₄ + 500 mg/kg PSMLE; Group E – CCl₄ + 100 mg/kg silymarin; Group F – No CCl₄ and no treatment.

**Figure 3.** Effects of oral administration of graded doses of PSMLE on liver weight and relative liver weight of albino rats given sub-acute toxic doses of CCl₄. [Group A – CCl₄ + 10 ml/kg distilled water (negative control), B - CCl₄ + 50 mg/kg PSMLE; Group C - CCl₄ + 250 mg/kg PSMLE; Group D - CCl₄ + 500 mg/kg PSMLE; Group E - CCl₄ + 100 mg/kg Silymarin; Group F – No CCl₄, No treatment (normal control)]

differences between Groups B, C, D and E in their CAT activity (Table 7). The SOD activity was also significantly ($p < 0.05$) lower in Groups A and B compared to other

groups (Table 7). Glutathione peroxidase (GPx) activity followed the same pattern as SOD with Groups A and B being significantly ($p < 0.05$) lower than Groups C, D, E

Table 7. Effects of oral administration of graded doses of PSMLE on *in vivo* oxidative stress and blood anti-oxidant marker levels of albino rats given sub-acute toxic doses of CCl₄.

Oxidative stress and antioxidant parameter	Means, with standard deviation in brackets					
	Group A	Group B	Group C	Group D	Group E	Group F
MDA (µmol/L)	17.61 (3.32) ^a	14.19(4.29) ^{ab}	9.65 (2.87) ^{bc}	7.52 (3.30) ^{cd}	7.65(2.88) ^{cd}	4.05 (1.90) ^d
CAT (IU/ml)	12.18(2.36) ^a	21.55(2.38) ^b	20.92 (3.35) ^b	20.35(3.49) ^b	23.14(4.40) ^{bc}	29.76(7.99) ^c
SOD (IU/ml)	0.221 (0.020) ^a	0.257(0.049) ^{ab}	0.300(0.024) ^c	0.278(0.020) ^{bc}	0.294 (0.027) ^{bc}	0.299 (0.019) ^c
GPx (IU/L)	82.50 (10.74) ^a	88.41(12.89) ^a	119.35 (20.17) ^b	124.29(23.90) ^b	127.16 (17.27) ^b	190.25(18.58) ^c
GSH (µg/µl)	0.016 (0.006) ^a	0.029(0.004) ^b	0.039(0.005) ^{bc}	0.041(0.005) ^{bc}	0.046 (0.006) ^c	0.068(0.015) ^d

^{a,b,c} Different alphabetical superscripts in a row indicate significant differences between the groups ($p < 0.05$). *Groups: Group A – CCl₄ + 10 ml/kg distilled water (negative control); Group B – CCl₄ + 50 mg/kg PSMLE; Group C – CCl₄ + 250 mg/kg PSMLE; Group D – CCl₄ + 500 mg/kg PSMLE; Group E – CCl₄ + 100 mg/kg silymarin; Group F – No CCl₄ and no treatment.

and F (Table 7). Treatment with PSMLE at all doses (50, 250, 500 mg/kg) and silymarin (100 mg/kg) (Groups B, C, D and E) led to significantly ($p < 0.05$) higher GSH levels when compared to the negative control (Group A) (Table 7). There was significant ($p < 0.05$) dose-dependent higher levels of GSH when all the PSMLE-treated groups were compared. However, there were significantly ($p < 0.05$) lower GSH levels in all the PSMLE-treated groups when compared with the normal control (Group F) (Table 7).

***In vitro* antioxidant activity of PSMLE**

The antioxidant activity of PSMLE at 10 µg/ml concentration was significantly ($p < 0.05$) lower than that of ascorbic acid (Figure 4). However, 50, 100, 200 and 400 µg/ml concentration of PSMLE produced significantly ($p < 0.05$) higher antioxidant activity when compared to the same concentrations of ascorbic acid (Figure 4). The PSMLE showed maximum anti-oxidant activity at 400 µg/ml and minimum activity at 10 µg/ml. There were no significant ($p > 0.05$) differences between the anti-oxidant activity values obtained at 50 µg/ml, 100 µg/ml, and 200 µg/ml concentrations (Figure 4). Also, there were no significant ($p > 0.05$) differences between the anti-oxidant activity values of the ascorbic acid standard at varied concentrations (Figure 4).

DISCUSSION

In the acute toxicity study, the rats tolerated the extract up to 5000 mg/kg, without any significant/adverse changes in their body and organ weights. This shows that the LD₅₀ is above 5000 mg/kg (OECD, 2001). An LD₅₀ above 5000 mg/kg is within the World Health Organization's category of substances "unlikely to present acute hazard in normal use" (WHO, 2001). This implies that PSMLE is safe for acute use in the treatment

of ailments and diseases for which it is effective (OECD, 2001). This concurs with earlier reports by Ihedioha et al. (2017; 2018) that methanol leaf extracts of *P. santalinoides* is not acutely toxic.

The phytochemicals observed in PSMLE are essential bioactive compounds commonly found in various herbs used for medicinal purposes. Similar compounds as recorded in this study have been identified in the methanol and ethanol extracts of the leaf and stem bark of *P. santalinoides* (Anowi et al., 2012; Eze et al., 2012; Odeh and Tor-Anyim, 2014; Enemali et al., 2019).

The higher serum ALT, AST and ALP activities in all the groups that were given CCl₄ showed that CCl₄ administration damaged the liver cells and altered the integrity of the hepatocytes (Boll et al., 2001; Kim et al., 2010). Elevation in serum transaminases (AST and ALT) is a biomarker of hepatocellular necrosis and hepatotoxicity (Friedman et al., 1996). The administration of PSMLE at the doses of 250 and 500 mg/kg led to significantly lower serum ALT activity, and at 250 mg/kg it led to significantly lower serum AST activity, and these were comparable to that of silymarin treatment (a known hepatoprotective drug). These findings are in agreement with the reports of Offor et al. (2015) who also recorded significantly lower ALT and AST activities in CCl₄-induced hepatotoxic albino rats treated with ethanol leaf extract of *P. santalinoides*. Reports by Ihedioha et al. (2017) also showed that methanol leaf extract of *P. santalinoides* restored hepatocellular integrity in acetaminophen-induced hepatotoxicity in albino rats. In another study on a related species of *Pterocarpus*, Ihedioha et al. (2019) also reported significantly lower serum ALT and AST activities in CCl₄-induced hepatotoxic albino rats treated with methanol leaf extract of *Pterocarpus mildbraedii* (a plant species in the same Genus *Pterocarpus*). The ability of the PSMLE to protect hepatocellular integrity from CCl₄-induced damage is believed to be due to its phytochemical constituents such as tannins, flavonoids and glycosides (Muriel et al., 2001; Ihedioha et al., 2017), which have been reported to be hepatoprotective. Other

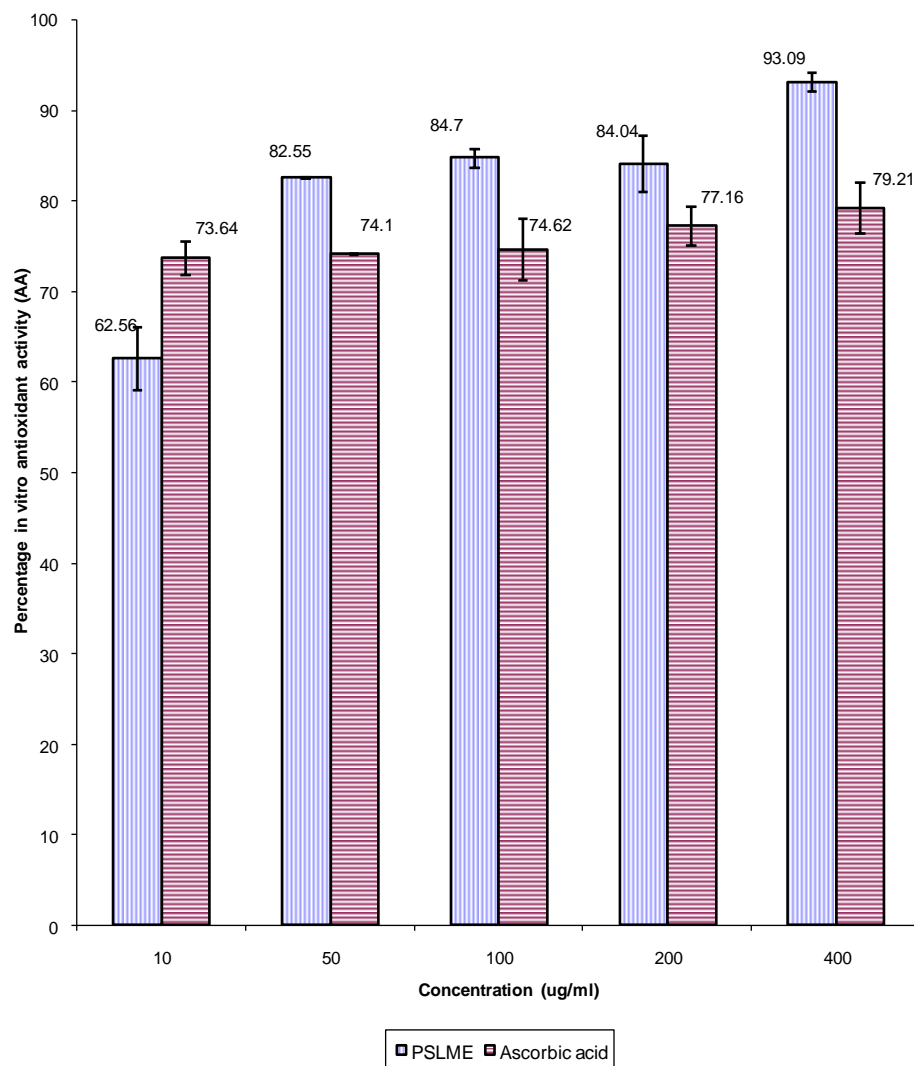


Figure 4. The *in vitro* antioxidant activity values of varied concentrations of PSLME and ascorbic acid using 1, 1-diphenyl 2 picryl hydrazyl (DPPH) assay.

natural products that possess similar phytochemical constituents have also been reported to protect against CCl₄-induced liver damage (Hsiao et al., 2003).

The significantly lower serum total proteins and serum globulin in the negative control group and other groups that were given CCl₄ are indications of the ability of CCl₄ to damage hepatocytes and impair protein synthesis (Navarro and Senior, 2006). Proteins form the major portion of dissolved substances in the plasma (Singh et al., 2011), and act as transport agents for a wide variety of substances such as hormones, lipids and vitamins. Proteins can be used as a supplementary test for hepatic biosynthetic functions (Friedman et al., 1996; Thapa and Walia, 2007; Singh et al., 2011). Decreased levels of proteins are found in liver disorders (Kipple, 2003). The

PSMLE (250 mg/kg) ameliorated the impaired protein synthetic function by leading to significantly higher serum total protein and serum globulins of the treated rats.

Elevation of serum globulin in CCl₄-induced hepatotoxic albino rats treated with *P. mildbraedii* (a plant of the same genus as *P. santalinoides*) methanol extract has also been reported (Ihedioha et al., 2019). Stimulation of protein synthesis is known to be a hepatoprotective mechanism. Protein synthesis accelerates the hepatocyte regeneration process and helps in the production of replacement liver cells (Rip et al., 1985; Tadeusz et al, 2001).

The significantly higher serum total cholesterol in the negative control group and other groups that were given CCl₄ are indications of alterations in serum lipid profile

caused by CCl₄ administration. The liver is the major site for the synthesis and clearance of lipoproteins; therefore, hepatotoxicity or damage to the hepatocytes can affect plasma lipids leading to alterations in lipid profile, and this has been found to be instrumental to the development of atherosclerosis (Ihedioha et al., 2013; Ihedioha et al., 2018). Hypercholesterolaemia occurs in hepatotoxicity as dyslipidaemia consequent upon dysfunction and alterations in hepatic lipid synthesis and clearance (Mandal et al., 2013). Treatment with PSMLE (500 mg/kg) and silymarin (100 mg/kg) ameliorated these effects by significantly lowering the serum total cholesterol of the treated albino rats at these doses. Ihedioha et al. (2017) also reported significantly lower serum total cholesterol levels in acetaminophen-induced hepatotoxic albino rats treated with methanol leaf extract of *P. santalinoides*.

Administration of CCl₄ also adversely affected bilirubin excretion as observed in all the groups that were given CCl₄. Bilirubin is an endogenous anion derived from the regular degradation of haemoglobin from the red blood cells. It is a chemical normally present in the blood in small amounts and excreted from the liver in form of bile. When the liver cells are damaged, they may not be able to excrete bilirubin in the normal way, thus causing a build-up of bilirubin in the blood and extracellular fluid (Singh et al., 2011). Increased levels of bilirubin may also result from decreased hepatic clearance and lead to jaundice and other hepatotoxicity symptoms (Saukkonen et al., 2006). Treatment with PSMLE (500 mg/kg) and silymarin (100 mg/kg) led to significantly lower serum total bilirubin in albino rats given toxic doses of CCl₄. They ameliorated the impaired excretory function induced by the CCl₄ administration, and enhanced hepatic clearance of bilirubin. Ihedioha et al. (2017) reported that *P. santalinoides* methanol extract significantly lowered total bilirubin level in acetaminophen-induced hepatotoxicity in albino rats. The ethanol extract of a related species (*Pterocarpus milbraedii*) had been reported by other researchers to significantly lower serum total bilirubin level in propanil and CCl₄-induced hepatotoxicity in albino rats (Otuochere and Farombi, 2015; Hamza et al., 2017; Ihedioha et al., 2019).

The relatively higher liver weights in albino rats given CCl₄ are indications of inflammation and/or degeneration which accompany CCl₄ toxicity (Tahashi et al., 2002; Bukhsh et al., 2014). Inflammation is a complex catalogue of vascular and tissue changes that develop as a response of tissue to injury (Ihedioha, 2003). Degeneration on the other hand, is a retrogressive change in tissues characterized by abnormal changes and decreases in function. Organs undergoing acute degenerative changes tend to be larger and heavier than normal (Ihedioha, 2003). Enlargement of the liver (hepatomegaly) is a common evidence of hepatic injury (Ihedioha and Chineme, 2005). Treatment with PSMLE at

all the doses used in the study and silymarin (100 mg/kg) significantly lowered this inflammation/degeneration-induced enlargement caused by CCl₄ toxicity and this suggests their amelioration of this inflammatory enlargement of the liver. Relatively lower liver weight has also been reported in hepatotoxic albino rats treated with *P. mildbraedii* methanol leaf extract (Ihedioha et al., 2019).

In the *in vivo* oxidative stress and antioxidants evaluation results, the higher doses of PSMLE (250 mg/kg and 500 mg/kg) were able to mop up more free radicals more than the lower dose (50 mg/kg). This implies that the PSMLE possesses anti-oxidant activity which is dose-related. Malondialdehyde (MDA) is one of the final products of the peroxidation of polyunsaturated fatty acids in cells and is a known marker of oxidative stress status in several diseases (Draper and Hadley, 1990; Gawel et al., 2004; DelRio et al., 2005). The MDA result in this present study concurs with the reports of Maruthupandian and Mohan (2011) on the lowering of plasma lipid peroxides in diabetic rats given ethanol extract of *Pterocarpus marsupium* wood and bark (a related plant belonging to the same genus). Administration of PSMLE enhanced catalase (CAT) activity. This is evident by the significantly higher catalase activity in all the groups treated with PSMLE. Catalase is a very important enzyme in protecting the cell from oxidative damage (Chelikani et al., 2004). It is the main enzyme that removes hydrogen peroxide (a reactive oxygen species), and blocks oxidative stress (Gaetani et al., 1996). The findings in this present study are similar to the reports by Maruthupandian and Mohan (2011) on the effects of a related plant of the same genus (*Pterocarpus marsupium*) on catalase activity of alloxan-induced diabetic rats. The superoxide dismutase (SOD) assay result in this present study is also an indication that the negative control (Groups A) and 50 mg/kg PSMLE-treated (Group B) rats had no significant protection from oxidative damage as compared to other groups. Treatment with PSMLE at the doses of 250 and 500 mg/kg (Groups C and D) effectively restored the SOD activity of these rats to a level comparable to that of normal rats not given CCl₄ (Group F). Superoxide dismutase (SOD) is an important anti-oxidant defense in living cells exposed to oxygen radicals. Earlier studies had shown that treatment with SOD decreased the generation of reactive oxygen species and oxidative stress (Fridovich, 1997; Gongora et al., 2006). Treatment with PSMLE at the doses of 250 and 500 mg/kg also restored the glutathione peroxidase (GPx) activity of the albino rats. The main biological role of GPx is to protect the organism from oxidative damage. Its biochemical function is to reduce lipid hydroperoxides to their corresponding alcohols and to reduce free hydrogen peroxide to water. Blood levels of GPx have been reported to be low in some important diseases, and had

significantly improved when antioxidants were administered (Lubos et al., 2011). The findings in this present study of significantly higher GPx activity in PSMLE-treated groups is comparable to the reports by Maruthupandian and Mohan (2011) on the effects of *P. marsupium* wood and bark extracts on GPx of alloxan-induced diabetic rats. Plasma total glutathione (GSH) is one of the major endogenous anti-oxidants produced by cells, which participates directly in the neutralization of free radicals and reactive oxygen species, as well as maintaining exogenous anti-oxidants such as vitamins C and E in their reduced (active) forms (Dringen, 2000). The higher total glutathione level recorded in this study for Group B (50 mg/kg PSMLE), Group C (250 mg/kg PSMLE), and Group D (500 mg/kg PSMLE) implies a dose-dependent anti-oxidant activity in these groups. Maruthupandian and Mohan (2011) also reported the enhancing effects by *P. marsupium* extracts on the plasma total glutathione of alloxan-induced diabetic rats, but their results did not show dose-dependence.

In vitro, the PSMLE evoked significant concentration-dependent antioxidant activity, from 50 to 400 µg/ml concentrations, suggesting that it could be of benefit in ameliorating tissue damaging effect of increased concentration of reactive oxygen species (ROS) seen in toxic liver diseases. This result concurs with the reports of Bothon et al. (2014), Kabine et al. (2015) and Akaniro-Ejim et al. (2018) who also reported that the aqueous and hydro-ethanol leaf and fruit extracts, and aqueous-ethanol leaf extract of *P. santalinoides* respectively, possess anti-oxidant activity *in vitro*.

The antioxidant properties of PSMLE may be attributed to its flavonoids, tannins and/or glycoside contents (Pietta, 2000; Hodek et al., 2002; Yi-Fang et al., 2002; Aruoma, 2003). The results obtained from the present study also concurred with the reports by Ihedioha et al. (2017), who attributed the ability of PSMLE in lowering serum ALT and AST activities to its antioxidant activity. Ihedioha et al. (2018) also reported that the lipid lowering capability of aqueous leaf infusion of *P. santalinoides* may be related to its antioxidant properties. This present result is also in agreement with reports by other researchers on the *in vitro* antioxidant activity of other species in the same genus *Pterocarpus*, such as *Pterocarpus marsupium* (Mohammadi et al., 2009; Tippani et al., 2010; Maruthupandian and Mohan, 2011), *Pterocarpus mildbraedii* (Nwozo et al., 2015), *Pterocarpus angolensis* (Traore et al., 2016) and *Pterocarpus erinaceus* (Patrick et al., 2016). These findings are significant because foods, spices and herbal formulations containing antioxidants are used pharmacologically to prevent, manage and /or treat diseases in which oxidative stress play critical roles such as liver diseases, heart diseases and diabetes mellitus (Sies, 1997; Halliwell and Gutteridge, 1989; Valko et al., 2007). The present study was limited to the evaluation of

the hepatoprotective and antioxidant activity of the crude methanol extract of the leaves of *P. santalinoides*. Further studies to elucidate the active fractions and pure compound(s) responsible for the hepatoprotective and antioxidant activity are ongoing, and shall be reported in future.

Conclusion

The findings in this present study confirm that extracts of the leaves of *P. santalinoides* significantly ameliorated hepatocellular injury and relieved oxidative stress, and may thus be beneficial in the treatment and management of toxic liver damage and diseases mediated by and/or associated with oxidative stress.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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