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ARTICLES

Safety and toxicity of aqueous leaf extracts of Camellia sinensis, Parquetina nigrescens and Telfairia occidentalis in mice 208

Dapsone in topical niosomes for treatment of acne vulgaris 221
Hatem Al Sabaa, Fatma M. Mady, Amal K. Hussein, Hossam M. Abdel-Wahab and Maha H. Ragaie
Plant extracts as potential phytotherapeutic products are supposed to be safe. However, adverse and untoward fatal effects have been reported. Study aimed to evaluate the safety and toxicity of aqueous extracts of Camellia sinensis, Parquetina nigrescens and Telfairia occidentalis leaves. The extracts were subjected to brine shrimp lethality bioassay and toxicities by Lorke’s method. Mice were given oral leaves extracts of C. sinensis (1000, 2000, 4000 mg/kg and 700, 1400, 2800 mg/kg); P. nigrescens (3000, 6000, 12000 mg/kg and 2000, 4000, 8000 mg/kg) and T. occidentalis (2500, 5000, 10,000 mg/kg and 1750, 3500, 7000 mg/kg) for acute and Sub-acute toxicity studies respectively. Toxicity was observed for the first 4hrs, then over a period of 24hrs and at least once daily for 14 days extended to 28 days. General behavior, adverse effects and mortality observed and evaluated throughout the experimental period. Camellia sinensis (LC50=418.6 µg/mL) with least toxicity on the brine shrimps compared to P. nigrescens (LC50=32.34 µg/mL) and T. occidentalis (LC50=8.32 µg/mL). LD50 of the extracts; 2800, 8000 and 7000 mg/kg for C. sinensis, P. nigrescens and T. occidentalis respectively. No death, No changes in body and relative organ weights. However, C. sinensis and T. occidentalis significantly increased in Haemoglobin (C. sinensis: 15.90 ± 0.33 (p < 0.00) and T. occidentalis: 14.67 ± 0.22 (p < 0.01)), PCV (C. sinensis: 46.20 ± 1.02 and T. occidentalis: 44.00 ± 0.71 (p < 0.00)), RBC (C. sinensis: 5.55 ± 0.12 and T. occidentalis: 5.49 ± 0.12 (p < 0.00)). No histomorphological changes in the vital organs except P. nigrescens with mild kidney interstitial fibrosis, mild glomerular hypercellularity and mild liver microhemorrhages. Various doses of the extracts did not cause mortality or serious signs of toxicity in mice.

Key words: Camellia sinensis, Parquetina nigrescens, Telfairia occidentalis, acute and sub-acute toxicities, No Observed Adverse Effect Level.
INTRODUCTION

Medicinal plants provide an alternative strategy in search for new drugs (Balunas and Kinghorn, 2005). There is an abundance of plants reputed in traditional medicine that possess protective and therapeutic properties (Farnsworth et al., 1985). Plants are leading compounds for the development of new medicines and also for the treatment and prevention of various human ailments (Farnsworth, 1994). It is likely that plants will continue to be a valuable source of novel molecules which may provide new and improved drugs (Fabricant and Farnsworth, 2001). Although, modern medicine has gradually developed in recent years, traditional medicine still receive high patronage (Ernst, 2000) since herbs and herbal medicines are believed to be effective, cheap and free from side effects (Bent, 2008). Thus, herbal remedies have remained as the basis for the development of new drugs (Koehn and Carter, 2005; Cragg and Newman, 2013).

Toxicity associated with herbal products (Bateman et al., 1998; Ernst, 1998) has alerted many national (Awodele, 2014) and international regulatory authorities (Saad et al., 2006) to develop and implement various sets of guidelines for assessing, monitoring, and preventing the toxicity associated with such herbal products (Chang, 1987). Toxicity tests are most widely used to examine specific adverse events or specific endpoints (Isbrucker et al., 2006) such as cancer, cardiotoxicity and skin/eye irritation, etc. Toxicity testing is also helpful in determining the “No Observed Adverse Effect Level (NOAEL)” dose and is helpful for further clinical trials (Setzer and Kimmel, 2003). Acute, sub-acute and chronic toxicity tests are routine safety tests carried out by pharmaceutical companies in the process of developing new medicines (Chang, 1987). However, in order to assess the toxic nature of compounds, acute oral toxicity is the first step to be carried out (Akhila et al., 2007).

Studies have shown that aqueous extract of Camellia sinensis, Telfairia occidentalis and Parqueztina nigrescens possess anti-mutagenic, anti-inflammatory, erythropoietic and effective chemo-preventive potentials against toxic chemicals and carcinogens. However, preliminary animal studies need to be done on these plant extracts as individual and in the combined form so as to provide scientific justification(s) for their consideration in clinical trials and thereby develop them as new radio-protectors and/or radio-mitigators in cancer radiotherapy. More so, the growing number of herbal medicine users globally (Elsenberg, 1998) and the lack of scientific data on the safety profiles of many herbal products however, have made it necessary to conduct safety studies on herbal products (Chang, 1987), thus justifying conducting this study.

Camellia sinensis

Green tea is a drink made from the steamed and dried leaves of Camellia sinensis, a shrub native to Asia (Costa, 2002). Green tea has been widely consumed in the Far East to promote good health for at least 3,000 years and is the second most consumed beverage in the world, with an estimated 18 to 20 billion cups consumed daily and an estimated average consumption of 1 L/person/day in the United Kingdom (Wu and Wei, 2002). This high rate of consumption may be justified possibly because of its biological properties such as: anti-oxidant (Feng et al., 2001), anti-obesity (Brown et al., 2011) and anti-cancer (Feng et al., 2001; Park et al., 2003; Sakata et al., 2004). The chemical composition of green tea is complex; it contains polyphenols, alkaloids (caffeine, theophylline, and theobromine), amino acids, carbohydrates, proteins, chlorophyll, volatile compounds, fluoride, minerals, trace elements and other undefined compounds. Among these, the polyphenols constitute the most interesting group that exhibit potent anti-oxidant activity in vitro and in vivo studies (Kondo et al., 2002). Although, tea has been considered a medicine and a health-derived beverage since ancient times, recently it has received a great deal of attention because the polyphenols are strong antioxidants (Feng et al., 2001; Sakata et al., 2004). Numerous studies have also demonstrated that the aqueous extract of the major tea polyphenols possesses anti-mutagenic (Sakata et al., 2004), anti-diabetic, anti-bacterial, anti-inflammatory, and lipid-cholesterol lowering properties (Brown et al., 2011; Olutunbosun et al., 2014).

Telfairia occidentalis (Cucurbitaceae)

This is an herbal plant cultivated mostly in the West African sub-region (Burkill, 1995). The leave extract of the plant is used locally in the treatment of malaria and anaemia (Gbile, 1986). Apart from its nutritional (Okoli and Mgbeogu, 1983), agricultural and industrial importance (Akoroda, 1990), the plant is also medicinally useful. It possesses anti-inflammatory (Oluwole et al., 2003), antibacterial (Odoemena and Essien, 1995), erythropoietic (Ajayi et al., 2000), anticholesterolemic (Eseyin et al., 2005a) and antidiabetic activities (Eseyin et al., 2005b, Ekpenyong et al., 2012). The ripe fruit *Corresponding author. E-mail: deluy008@yahoo.com, deluy008@gmail.com. Tel: 08030467510.

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contains up to 13% oil. The leaves and the young shoots of the plant are frequently eaten as a potherb (Ajao and Akindele, 2013). The seeds of the plant are also popular items of diet and are cooked whole and ground up into soups (Dina et al., 2006). The leaves also contain protein, vitamins, and flavours (Ekpenyong et al., 2012; Ajao and Akindele, 2013). In Nigeria, the herbal preparation of the plant has been employed in the treatment of sudden attack of convulsion, malaria and anaemia (Dina et al., 2006).

**Parquetina nigrescens (Periplocaceae)**

This is a shrub found in Equatorial West Africa and has been in use in traditional medicine for centuries (Burkett, 1968). In Oyo State Nigeria, the leaves have been reputed for treatment of helmintiasis (intestinal worm) while the roots are reputed for use as an antirheumatic [oral communications]. Over the years, the leaf and root decoctions of *P. nigrescens* have been used for the treatment of gonorrhea and menstrual disorders (Schlage, 2002). The whole plant is used to stupefy fish in Ghana and Liberia, while the leaves and latex are used for the treatment of rickets, diarrhoea, skin lesions and tropical skin diseases (Schlage, 2002). The leaves of the plant have been used for the treatment of wounds, boils, and carbuncles in Africa (Agyare et al., 2009). *P. nigrescens* is also a constituent of a commercial herbal preparation (Jubi formular®) in Nigeria which is used in the treatment of sickle cell anaemia in human (Imaga et al., 2010). The Jubi formular was shown to restore decreased haematocrit and haemoglobin concentration in *Trypanosoma brucei* induced anaemia (Erah et al., 2003). Similarly, anti-sickling property of the root and leaves of *P. nigrescens* was confirmed by Kade et al. (2003) while the whole plant was investigated by Imaga et al. (2010). Agbor et al. (2001) also investigated and confirmed the antianaemic activity of aqueous extracts of *P. nigrescens* leaf on haemorrhagic anaemia induced in rats (Agbor and Odetola, 2001). Also, Akinyemi and Dada (2013, 2014) reported the anti-typhoid activity of ethanolic leaf extract of *P. nigrescens* in mice (Akinyemi and Dada, 2013; Akinyemi and Dada, 2014). The methanol leaf and other aerial parts extracts, and root extract of *P. nigrescens* have been shown to exhibit dose and time dependent toxicity in animals (Louis Adu-Amoah et al., 2014; Owoye et al., 2011).

Although, several investigations have been conducted on *C. sinensis*, *T. occidentalis* and *P. nigrescens* leaves as foods and herbal remedies, there is dearth of information on the safety evaluation of these plants as biological agents for radio-protection in experimental models. This study therefore, seeks to evaluate the acute and sub-acute toxicity potentials of aqueous leaves extracts of *C. sinensis*, *P. nigrescens* and *T. occidentalis* in mice.

**MATERIALS AND METHODS**

**Materials, apparatus and reagents**

These includes analytical balance (Golden-Mettler, U. S. A.), normal saline, formalin buffer, fixative (4%) paraformaldehyde, heamatoxylin/eosin, oral cannula, eppendorf, spatula, syringes (1, 2, 5, and 10 mL), laboratory wares and consumables.

**Collection and identification of the plant material**

Fresh leaves of *P. nigrescens* and *T. occidentalis* were collected from the Plant Garden of African Centre for Herbal Research Institute, University of Ilorin while a refined product of *C. sinensis* was purchased from pharmaceutical premises in Ilorin. The plants were identified and authenticated by a taxonomist of the Department of Plant Biology, University of Ilorin, Nigeria. *P. nigrescens* was given Serial Number 876 and Ledger Number 67 while *T. occidentalis* was given Serial Number 959 and Ledger Number 150. Thereafter, collected samples were deposited in the herbarium of the institution for future reference.

**Extraction of the plants materials**

Four hundred grams and 350 g of the powdered leaves of *T. occidentalis* and *P. nigrescens* respectively were each soaked in distilled water in a closable container. The finished product (fine granules) of *C. sinensis* was also weighed (500 g) and soaked in distilled water. These were shaken for about 5 min and left to extract by means of maceration (shaking the mixture intermittently) at 28°C for 72 h. The mixtures were filtered into a porcelain crucible using a fine mesh. The supernatant was concentrated below 40°C using rotary evaporator and then freeze-dried. The extract was stored at 4°C in freeze-dried form and used for the toxicity experiments later.

**Preliminary phytochemical screening**

The phytochemical constituents of the aqueous extract were determined using standard procedures described by Sofowora (2008) and Trease and Evans (2009). The extracts were tested for the presence or absence of saponins, tannins, alkaloids, anthraquinones, cardiac glycosides, flavonoids and terpenoids.

**Brine shrimp lethality (BSL) bioassay**

The eggs of Brine shrimp (*Artemia salina*) were obtained from Pharm., Kayode M. Salawu and hatched in natural seawater obtained from the Bar Beach, Ikoyi, Lagos and incubated for 48 h in 3.8 g/L seawater. The assay was the method described by McLaughlin (1991). Ten milligrams (10 mg) of three extracts were diluted to 1000 µg/mL by adding the sea water. Serial dilutions of the extracts were made in 96-well microplates in triplicates. Negative control wells contained sea water, while cyclophosphamide was used as the positive control. A 250 µL suspension of nauplii in the extract was added to each well. The plates were incubated at room temperature (25°C) for 24 h. The number of dead nauplii in each well was counted.

Data obtained was analyzed by computer program (Graphpad prism version 6.00). The concentration with 50% lethality (LC50) was calculated by nonlinear regression analysis.
Ethical approval for animal studies

Ethical clearance and approval for the toxicity studies in mice was given by the University of Ilorin Ethical Review Committee, Ilorin, Nigeria in accordance with the Guide for Care and Use of Laboratory Animals, NIH, Department of Health Services Publication, USA, no. 83-23, revised 1985.

Animals

Healthy Swiss male albino mice (17-27 g) were selected for both the acute and sub-acute toxicity studies and kept in in plastic cages (34 × 47 × 18 cm³) in the animal room of the Toxicology Unit, Department of Pharmacology and Toxicology, Faculty of Pharmaceutical Sciences, University of Ilorin, Ilorin, Nigeria. The animals were kept in an air conditioned environment with three mice in each cage and maintained at room temperature of 25 ± 2°C with relative humidity (60% ± 10%) under 12 h night and light cycle. The animals used for the experiment were approved by Animal Ethics Committee of the University. They had free access to standard pellets as basal diet and water ad libitum. Animals were habituated to laboratory conditions for two weeks prior to experimental protocol to minimize if any of non-specific stress. All experimental procedures were conducted as stipulated by the Committee as well internationally accepted guidelines for laboratory animal use and care.

Acute toxicity procedure

Acute toxicity studies of aqueous extracts of *C. sinensis*, *P. nigrescens* and *T. occidentalis* were carried out in male mice using Lorke’s method (n=3). Twenty-seven mice were grouped into equal nine groups. Following an overnight fast, the mice were weighed and the doses were calculated in reference to their body weights. The first three groups received aqueous extract of *C. sinensis* (500, 1000 and 2000 mg/kg), the second three groups received aqueous extracts of *P. nigrescens* (1000, 2000 and 4000 mg/kg) while the third three groups received the aqueous extract of *T. occidentalis* (1250, 2500 and 5000 mg/kg). All the mice were observed for general behavioral changes; symptoms of toxicity and mortality after treatment for the first four (critical) hours, then further observations were made every 8 h for 24 h. The absence of death of any animal in this phase was a pre-requisite to proceed to the second phase.

In the second phase, 9 mice were grouped into three of one mouse each. The first group received aqueous extract of *C. sinensis* (1000, 2000 and 4000 mg/Kg), the second group received aqueous extracts of *P. nigrescens* (3000, 6000 and 12000 mg/Kg) while the third group received the aqueous extract of *T. occidentalis* (2500, 5000 and 10000 mg/Kg). The animals were observed critically for about 30 min for signs of toxicity or mortality and further observations were made every 8 h for 24 h. Further critical observation of all the mice were made for a period of 14 days.

Sub-acute toxicity studies

Sub-acute toxicity study (28-day repeated oral toxicity study) was carried out based on the calculated LD₅₀. Mice were divided into ten groups with 5 animals per group. Group I received distilled water orally at a dose of 10 ml/kg body weight and served as the control group whereas Groups II, III and IV received *C. sinensis* at dose rates of 700, 1400 and 2800 mg/kg respectively. Groups V, VI and VII received *P. nigrescens* at dose rate of 2000, 4000 and 8000 mg/kg respectively, while Groups VIII, IX and X received *T. occidentalis* at 1750, 3500 and 7000 mg/kg body weight respectively. All the groups of mice were observed twice daily for mortality and morbidity till the completion of the experiment. All the animals were observed for clinical signs and the time of onset and the duration of these symptoms were recorded. Body weights of the mice in all groups were recorded once before the start of dosing, once weekly during the treatment period and finally on the day of sacrifice. The amount of food intake was recorded every day and the data were expressed as 7 days cumulative value. At the end of the experiment (on the 29th day), following an overnight fast of 8 h (only water allowed) prior to necropsy and blood collection, all animals in various groups were anaesthetized under chloroform prior to euthanization and then decapitated by cervical dislocation.

Blood samples were collected by cardiac puncture into ethylene diamine tetra acetic acid (EDTA-2K) and plain containers for haematological and biochemical investigations respectively. Blood in plain containers were allowed to clot and centrifuged and serum was collected for biochemical analyses.

Hematological parameters

EDTA blood was used for the measurement of hemoglobin, red blood cell count, white blood cell count, platelet count using fully automated haematology analyzer (Sysmex KX 21N).

Biochemical parameters

The serum was used to measure sodium, potassium [Ion Selective Electrodes], while urea, creatinine, alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), triglycerides, total cholesterol and total protein were determined using fully automated biochemical analyzer (Chemray 240, India).

Histopathology

Following blood collection on day 29, all the animals were euthanized for gross pathological examinations of all major internal organs. Organs of interest [liver, kidney, brain, stomach, heart, testis, lung and spleen] were dissected, immediately cleaned of blood using physiological saline, weighed and preserved in 10% neutral buffered formalin. Tissues embedded in paraffin wax were sectioned 5 μm thick, stained with haematoxylin and eosin, mounted on glass slides and examined under a standard light microscope for histopathological study.

Statistical analysis

Results were expressed as mean ±standard error of mean (SEM). Data obtained was analyzed using one way ANOVA followed by Tukey HSD. P values <0.05 was considered as statistically significant.

RESULTS

All the three aqueous extracts tested showed the presence of saponins, tannins, alkaloids, cardiac glycosides, flavonoids, and terpenoids. However, anthraquinones (both free and combined form) are present in *P. nigrescens* and *T. occidentalis* but absent in
Table 1. Preliminary phytochemical screening.

<table>
<thead>
<tr>
<th>Bioactive constituent</th>
<th>Chemical test</th>
<th>Telfairia occidentalis</th>
<th>Parquetina nigrescens</th>
<th>Camellia sinensis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alkaloid</td>
<td>Drangendorff's</td>
<td>+</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td></td>
<td>Wagner's</td>
<td>+</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td></td>
<td>Meyer's</td>
<td>+</td>
<td>+</td>
<td>+++</td>
</tr>
<tr>
<td>Flavonoid</td>
<td>Shinoda's test</td>
<td>++</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td></td>
<td>Lead Ace Tate</td>
<td>++</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>Tannins</td>
<td>FeCl₃</td>
<td>+</td>
<td>+</td>
<td>+++</td>
</tr>
<tr>
<td></td>
<td>Frothing</td>
<td>++</td>
<td>+</td>
<td>+++</td>
</tr>
<tr>
<td>saponins</td>
<td>Emulsifying</td>
<td>++</td>
<td>+</td>
<td>+++</td>
</tr>
<tr>
<td></td>
<td>Haemolysis</td>
<td>+++</td>
<td>+</td>
<td>+++</td>
</tr>
<tr>
<td>Anthraquinone</td>
<td>Free</td>
<td>+</td>
<td>+</td>
<td>_</td>
</tr>
<tr>
<td></td>
<td>Combined</td>
<td>++</td>
<td>+</td>
<td>_</td>
</tr>
<tr>
<td>Cardiac Glycoside</td>
<td>Keller Killiani</td>
<td>+++</td>
<td>+++</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Kedde</td>
<td>++</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>Steriod Terpenoid</td>
<td>Salkowski</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
</tr>
</tbody>
</table>

*, Absence of component; +, Trace presence of component; ++, moderate amount of component, ++++, Copious amount of component.

C. sinensis as shown in Table 1.

**Acute toxicity studies**

Oral administration of extracts of C. sinensis (500, 1000 and 2000), P. nigrescens (1000, 2000 and 4000 mg/kg), T. occidentalis (1250, 2500 and 5000 mg/kg) produced no deaths or clinical signs of toxicity in mice. However, there were mortality and clinical signs of toxicity at 4000, 12,000 and 10000 mg/kg body weight for C. sinensis, P. nigrescens and T. occidentalis respectively. The LD₅₀ for C. sinensis, P. nigrescens and T. occidentalis extracts were 2828.43, 8485.28 and 7071.07, mg/kg respectively. Thus, convenient doses were chosen for sub-acute toxicity studies to preclude the lethal range.

**Sub-acute toxicity studies**

There were no treatment related toxicity signs or mortality observed among mice treated at 700, 1400 and 2800 mg/kg for C. sinensis; 2000, 4000 and 8000 mg/kg for P. nigrescens and 1750, 3500 and 7000 mg/kg for T. occidentalis during the 4 weeks of treatment.

**DISCUSSION**

Medicinal plants were and are still one of the major sources of modern medicine. The general belief that herbal products prepared from medicinal plants are safe since they are from natural sources are not always correct. Interest in medicinal plant's pharmacognosy has increased due to trend of phytotherapy as alternative to orthodox medicine. With the increased interest in the pharmacological activities of the medicinal plants, there is a reason for thorough scientific investigations of these medicinal plants for efficacy and potential toxicity. Proper scientific evidence is necessary to establish toxicological profile of commonly used medicinal plants as safe, nontoxic and pharmacologically active. Acute and sub-acute safety evaluation of plants extracts are required as an effective parameter for calculating the therapeutic index of drugs and chemicals, to identify the further range of doses in animal studies and to explain the probable clinical signs evoked by the test compounds under investigation (Akhila et al., 2007). Results obtained from toxicity studies on animals will be critical for positive judgement on the safety of medicinal plants if they are found to have adequate potential for development into pharmacological compounds (Chang, 1987). Against this background, the present study evaluated the acute and sub-acute toxicity of aqueous leaves extracts of C. sinensis, P. nigrescens and T. occidentalis in mice using standard toxicological procedures. Several studies however, conducted on these plants have established numerous medicinal properties and health benefits. Aqueous leaves extracts of the three plants have well-documented evidence-based findings of promotive, preventive, corrective and curative potentials. Towards
Table 2. Brine shrimp lethality of *C. sinensis*, *P. nigrescens* and *T. occidentalis* extracts.

<table>
<thead>
<tr>
<th>Concentration (µg/mL)</th>
<th>Percentage lethality</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><em>C. sinensis</em></td>
</tr>
<tr>
<td>1000</td>
<td>100</td>
</tr>
<tr>
<td>500</td>
<td>100</td>
</tr>
<tr>
<td>100</td>
<td>10</td>
</tr>
<tr>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>LC₅₀ (µg/mL)</td>
<td>418.6</td>
</tr>
</tbody>
</table>

achieving their safety profiles, gross behavioural assessment; body/organ weight; food intake; haematological; and biochemical parameters; and histomorphology of major vital organs were evaluated.

The preliminary phytochemical assay was conducted to determine the presence of plant metabolites in all the three samples under study. The three aqueous extracts tested depicted that none of them contains toxic plant metabolites rather, tannins, alkaloids, cardiac glycosides, flavonoids, saponins, and terpenoids were present (Tables 1, 3, 4 and 5). Since all the secondary metabolites present are non-toxic type, perhaps this justified the absence of any significant morbidity and lethality in all the groups in both acute and sub-chronic toxicity studies. Otherwise, significant morbidity and lethality might have been witnessed if compounds like anthocyanins were to be present, even though the mild toxic signs observed in groups administered high doses of *T. occidentalis* (10000 mg/kg) and *P. nigrescens* (12,000 mg/kg) may likely be attributed to the copious presence of anthraquinones (both free and combined form) in *P. nigrescens* and *T. occidentalis*, respectively.

Brine shrimp lethality (BSL) model has proved helpful as a preliminary screening model in the drug design and synthesis of cytotoxic compounds (Nazir et al., 2013). The lethality of the extracts on the brine shrimps was classified according to the method of Padmaja et al. (2002). Where LC₅₀ ≥ 1000 µg/mL was considered to be non-toxic, LC₅₀ = 500 to 1000 µg/mL as weakly toxic, LC₅₀ = 100 to 500 µg/mL as moderately toxic and LC₅₀ ≤ 100 µg/mL as strongly toxic. Only extract of *C. sinensis* (LC₅₀=418.6 µg/mL) was slightly more toxic on the brine shrimps, while *P. nigrescens* (LC₅₀=32.34 µg/mL) and *T. occidentalis* (LC₅₀=8.32 µg/mL) were observed to be strongly toxic on the brine shrimps (Table 2).

The LD₅₀ of the extract were low for *C. sinensis*, but high for *P. nigrescens* and *T. occidentalis* (2828.43, 8485.28 and 7071.07 mg/kg respectively). Following the establishment of LD₅₀ for each of the plant, the sub-acute dose ranges of low, moderate and high doses were assigned to each of the extract (Tables 3 to 5). The results showed no significant treatment-related signs or deaths in the graduated doses in all the plant: *C. sinensis* (700, 1400 and 2800 mg/kg), *P. nigrescens* 2000, 4000 and 8000 mg/kg) and *T. occidentalis* (1750, 3500 and 7000 mg/kg) throughout the 28-day of the administration. The *T. occidentalis* group showed no signs of acute toxicity, rather they exhibited calm behavior and ptosis. This finding is similar to the finding of Ekpenyong (2012). Contrarily, the animals administered doses of *C. sinensis* extract showed signs of hyperactivity, irritable behavior, and agitation within 2 h post-administration, however, with no clinical signs of toxicity. Behavioral manifestations observed for 2 h post-oral treatment of all dose levels of aqueous leaf extract of *P. nigrescens* included reduced locomotion, calmness, writhing effects, passivity and hypovigour.

Generally speaking, there were no statistically significant differences recorded in body weight, food intake and relative organ weights in all the study groups. However, food intake was observed to decrease proportionately with decrease body weight when compared with the control group, albeit not statistically significant (Figure 1 to 3). These findings of poor food intake and reduced body weight may probably be attributed to the low intestinal uptake (bioavailability) of the extracts and green tea (*C. sinensis*) in particular because absorption of *C.* is improved on an empty stomach as previously reported by Chen et al. (1997) and Naumovski et al. (2015). It could also support the notion that green tea can be more effective in reducing weight insome populations (Brown et al., 2011).

Further, treatment with aqueous leaf extracts for 28 days significantly increase haemoglobin, red blood cell counts and packed cells volume as observed in groups administered *C. sinensis* and *T. occidentalis* but, contrary to the group given *P. nigrescens* when compared to the control group (Tables 6 to 8). Similarly, there were significant increments in leucopoiesis in groups given moderate and high doses of *C. sinensis* and in low and moderate doses of *T. occidentalis* and *P. nigrescens* (Tables 6 to 8). Megakaryopoiesis was also significantly increased in low and moderate doses of *C. sinensis* and *T. occidentalis*, in agreement with previous reports (Eseyin et al., 2003a; Park et al., 2003; Sakata et al., 2004; Dina et al., 2006).

Transaminases (AST and ALT) are well known good indicators of liver function and are used as biomarkers to show probable toxicities of drugs and xenobiotics.
Figure 1. Mean of the effect of aqueous leaves extract of *Camellia sinensis* on food intake (g), body weight gain (kg) and relative organ weight (g) in mice.

Figure 2. Mean of the effect of aqueous leaves extract of *Telfairia occidentalis* on food intake (g), body weight gain (kg) and relative organ weight (g) in mice.

Figure 3. Mean of the effect of aqueous leaves extract of *Parquetina nigrescens* on food intake (g), body weight gain (kg) and relative organ weight (g) in mice.

Usually, destruction of the liver parenchymal cells results in an increase in both AST/ALT enzymes in the blood (Odoemena et al., 1995; Ajayi et al., 2000). In this study however, no significant changes in the ALT/AST/ALP and total protein levels were reported in various doses of the extracts. Nonetheless, significant increase in AST/ALT was observed in the group administered high dose of *P. nigrescens* only. Although, cholesterol has not significantly changed, triglycerides were significantly lowered with low doses of *C. sinensis* and high dose of *P. nigrescens*.
Table 3. Acute oral toxicity of leaves aqueous extracts of *C. sinensis*.

<table>
<thead>
<tr>
<th><em>C. sinensis</em></th>
<th>Phase 1</th>
<th>Phase 2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Dose (mg/kgbw)</td>
<td>Mortality</td>
</tr>
<tr>
<td>Low</td>
<td>500</td>
<td>0/3</td>
</tr>
<tr>
<td>Moderate</td>
<td>1000</td>
<td>0/3</td>
</tr>
<tr>
<td>High</td>
<td>2000</td>
<td>0/3</td>
</tr>
<tr>
<td><strong>LD$_{50}$</strong></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 4. Acute oral toxicity of leaves aqueous extracts of *P. nigrescens*.

<table>
<thead>
<tr>
<th><em>P. nigrescens</em></th>
<th>Phase 1</th>
<th>Phase 2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Dose (mg/kgbw)</td>
<td>Mortality</td>
</tr>
<tr>
<td>Low</td>
<td>1000</td>
<td>0/3</td>
</tr>
<tr>
<td>Moderate</td>
<td>2000</td>
<td>0/3</td>
</tr>
<tr>
<td>High</td>
<td>4000</td>
<td>0/3</td>
</tr>
<tr>
<td><strong>LD$_{50}$</strong></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 5. Acute oral toxicity of leaves aqueous extracts of *T. occidentalis*.

<table>
<thead>
<tr>
<th><em>T. occidentalis</em></th>
<th>Phase 1</th>
<th>Phase 2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Dose (mg/kgbw)</td>
<td>Mortality</td>
</tr>
<tr>
<td>Low</td>
<td>1250</td>
<td>0/3</td>
</tr>
<tr>
<td>Moderate</td>
<td>2500</td>
<td>0/3</td>
</tr>
<tr>
<td>High</td>
<td>5000</td>
<td>0/3</td>
</tr>
<tr>
<td><strong>LD$_{50}$</strong></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*P. nigrescens*. Electrolytes ($\text{Na}^+$ and $\text{K}^+$) were not significantly affected in all the groups except with the group administered high doses of *T. occidentalis* and *P. nigrescens*. Nevertheless, urea and creatinine were not significantly affected in all the groups (Tables 9 to 11).

Moreover, histopathological studies provide supportive evidence for biochemical and haematological observations. Overall histopathological examinations and analyses of the major vital organs which include; liver, kidney, heart, lungs, stomach, spleen and testes showed a well preserved cytoarchitecture. Specific features were assessed which include but not limited to scarring (cirrhosis), necrosis, inflammation, sinusoidal dilatation, haemorrhage, hypercellularity, fibrosis, lymphocytic infiltration. Various doses of all the groups did not only show no toxicity but equally showed well preserved tissue morphology. Notwithstanding, *P. nigrescens* at high doses showed mild kidney interstitial fibrosis, mild glomerular hypercellularity (mesangial proliferation), mild inflammation of the intestines, microhemorrhages and mild liver changes (mild inflammation, mild central vein fibrosis, mild central vein inflammation).

Similarly, testes were well preserved with complete sequence of seminiferous tubules, normal germ cells, normal leydig cells as well intact basement membrane. These mild tissues derangement corroborated the biochemical findings of slightly increased values of AST and ALT observed in this group. Previous study of ethanolic extracts of *P. nigrescens* in rats reported renal haemorrhage and inflammation and hepatic inflammation in low doses, while high dose administration showed restoration of glomerular tufts and improved hepatic vasculature with reduced inflammatory infiltrates (Feng et al., 2001). This however, contradicts the study findings since groups administered various doses of *P. nigrescens* did not reveal any end organ toxicity as assessed by histological and biochemical analyses. Also, Ajao and Akindele (2013) reported no signs of delayed toxicity or death with very high oral doses of *T. occidentalis* which is similar to reports made by Chow et al. (2003), Yu et al. (2011) and Ekpenyong et al. (2012). However; administration of high doses either Teavigo or Polyphenon E (two brands of green tea catechins) to beagle dogs resulted in dose-dependent toxicity with vomiting and diarrhea, resulting in death. It is noteworthy that, eagle dogs have better absorption rates of green
Table 6. Mean±SEM of the effect of varying doses of aqueous leaf extract of *C. sinensis* on haematologic parameters in mice.

<table>
<thead>
<tr>
<th>Group</th>
<th>Hb (g/dl)</th>
<th>PCV (%)</th>
<th>RBC×10^{12}</th>
<th>MCV (fl)</th>
<th>MCH (pg)</th>
<th>MCHC (g/dl)</th>
<th>WBC × 10^9</th>
<th>PLT × 10^12</th>
<th>NEUT (%)</th>
<th>LYMP (%)</th>
<th>EOSIN (%)</th>
<th>MONO (%)</th>
<th>BASO (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>12.00±0.22</td>
<td>36.00±0.71</td>
<td>4.42±0.12</td>
<td>84.20±2.76</td>
<td>28.80±0.86</td>
<td>34.20±0.49</td>
<td>3.56±0.14</td>
<td>347.80±19.58</td>
<td>11.40±1.72</td>
<td>86.40±1.86</td>
<td>2.20±0.66</td>
<td>0.00±0.00</td>
<td>0.00±0.00</td>
</tr>
<tr>
<td>700 mg/kg</td>
<td>14.88±0.55*</td>
<td>43.20±1.62*</td>
<td>5.25±0.17</td>
<td>87.60±2.71</td>
<td>27.40±0.98</td>
<td>32.40±1.08</td>
<td>4.28±0.15</td>
<td>421.20±12.03*</td>
<td>16.20±2.62</td>
<td>81.20±1.35</td>
<td>2.20±0.49</td>
<td>0.60±0.40</td>
<td>0.00±0.00</td>
</tr>
<tr>
<td>1400 mg/kg</td>
<td>15.90±0.33*</td>
<td>46.20±1.02*</td>
<td>5.55±0.12*</td>
<td>92.40±1.86</td>
<td>30.80±2.20</td>
<td>31.60±1.03</td>
<td>5.96±0.25*</td>
<td>457.00±17.78*</td>
<td>9.20±0.49</td>
<td>88.80±0.49</td>
<td>2.00±0.05</td>
<td>0.00±0.00</td>
<td>0.00±0.00</td>
</tr>
<tr>
<td>2800 mg/kg</td>
<td>13.70±0.27*</td>
<td>41.80±0.80*</td>
<td>5.06±0.08*</td>
<td>83.60±2.94</td>
<td>28.00±1.14</td>
<td>33.40±1.12</td>
<td>5.38±0.49*</td>
<td>406.00±12.84</td>
<td>17.80±1.50</td>
<td>78.80±1.48</td>
<td>2.60±0.40</td>
<td>0.60±0.00</td>
<td>0.20±0.20</td>
</tr>
</tbody>
</table>

The Hb, PCV and RBC were significantly increased in all the doses with moderate dose showing the highest value. WBC was significantly increased at moderate and high doses, PLT significantly increased at low and moderate doses. Values are expressed as Mean±SEM (n=5). * indicates significant difference when compared to Control, at p<0.05.

Table 7. Mean±SEM of the effect of varying doses of aqueous leaf extract of *T. occidentalis* on haematologic parameters in mice.

<table>
<thead>
<tr>
<th>Group</th>
<th>Hb (g/dl)</th>
<th>PCV (%)</th>
<th>RBC×10^{12}</th>
<th>MCV (fl)</th>
<th>MCH (pg)</th>
<th>MCHC (g/dl)</th>
<th>WBC × 10^9</th>
<th>PLT × 10^12</th>
<th>NEUT (%)</th>
<th>LYMP (%)</th>
<th>EOSIN (%)</th>
<th>MONO (%)</th>
<th>BASO (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>12.00±0.22</td>
<td>36.00±0.71</td>
<td>4.42±0.12</td>
<td>84.20±2.76</td>
<td>28.80±0.86</td>
<td>34.20±0.49</td>
<td>3.56±0.14</td>
<td>347.80±19.58</td>
<td>11.40±1.72</td>
<td>86.40±1.86</td>
<td>2.20±0.66</td>
<td>0.00±0.00</td>
<td>0.00±0.00</td>
</tr>
<tr>
<td>1250 mg/kg</td>
<td>14.41±0.52*</td>
<td>42.60±1.86*</td>
<td>5.22±0.11*</td>
<td>83.80±1.72</td>
<td>29.20±0.80</td>
<td>34.00±0.63</td>
<td>4.98±0.28*</td>
<td>442.60±8.14*</td>
<td>12.60±1.60</td>
<td>80.40±1.75</td>
<td>1.40±0.25</td>
<td>0.00±0.00</td>
<td>0.00±0.00</td>
</tr>
<tr>
<td>2500 mg/kg</td>
<td>14.67±0.22*</td>
<td>44.00±0.71*</td>
<td>5.49±0.12*</td>
<td>91.60±1.75</td>
<td>29.20±0.58</td>
<td>31.40±1.12</td>
<td>6.50±0.16*</td>
<td>485.20±8.49*</td>
<td>11.60±1.03</td>
<td>86.80±1.24</td>
<td>1.60±0.40</td>
<td>0.00±0.00</td>
<td>0.00±0.00</td>
</tr>
<tr>
<td>5000 mg/kg</td>
<td>14.20±0.42*</td>
<td>41.80±0.80*</td>
<td>5.07±0.10*</td>
<td>82.80±2.40</td>
<td>29.20±0.58</td>
<td>34.00±0.68</td>
<td>4.50±0.22*</td>
<td>431.60±21.01*</td>
<td>13.60±2.46</td>
<td>86.40±2.65</td>
<td>2.00±0.32</td>
<td>0.00±0.00</td>
<td>0.00±0.00</td>
</tr>
</tbody>
</table>

The Hb, PCV, RBC were significantly increased in all the doses. WBC and PLT were significantly increased consistently at low and moderate doses with moderate doses showing the highest value. Values are expressed as Mean±SEM (n=5). * indicates significant difference when compared to control, at (p<0.05).

Table 8. Mean±SEM of the effect of varying doses of aqueous leaf extract of *C. sinensis* on biochemical parameters in mice.

<table>
<thead>
<tr>
<th>Group</th>
<th>Na (mmol/L)</th>
<th>K (mmol/L)</th>
<th>ALP (mmol/L)</th>
<th>ALT (mmol/L)</th>
<th>AST (mmol/L)</th>
<th>UREA (mmol/L)</th>
<th>CREAT. (mmol/L)</th>
<th>CHOL. (mmol/L)</th>
<th>TRIGLY (mmol/L)</th>
<th>T.PROTEIN (mmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>135.80±3.90</td>
<td>7.20±0.13</td>
<td>51.80±2.48</td>
<td>145.20±3.28</td>
<td>228.80±7.53</td>
<td>2.40±0.16</td>
<td>34.00±1.30</td>
<td>1.58±0.11</td>
<td>0.90±0.05</td>
<td>50.60±1.91</td>
</tr>
<tr>
<td>700 mg/kg</td>
<td>132.80±1.36</td>
<td>6.82±0.28</td>
<td>43.00±1.52</td>
<td>133.00±1.62</td>
<td>206.20±3.80</td>
<td>2.50±0.15</td>
<td>33.60±1.57</td>
<td>1.58±0.07</td>
<td>0.58±0.07</td>
<td>43.60±1.63</td>
</tr>
<tr>
<td>1400 mg/kg</td>
<td>143.40±1.54</td>
<td>7.02±0.22</td>
<td>46.20±1.28</td>
<td>143.20±2.22</td>
<td>208.20±2.76</td>
<td>2.26±0.12</td>
<td>33.40±1.08</td>
<td>1.62±0.09</td>
<td>0.80±0.03</td>
<td>49.20±1.39</td>
</tr>
<tr>
<td>2800 mg/kg</td>
<td>147.00±3.32</td>
<td>6.90±0.07</td>
<td>46.20±1.36</td>
<td>133.20±1.93</td>
<td>215.40±3.69</td>
<td>2.28±0.10</td>
<td>33.20±1.39</td>
<td>1.58±0.09</td>
<td>0.70±0.05</td>
<td>44.40±1.33</td>
</tr>
</tbody>
</table>

The parameters were not statistically significant except significant decrease in Triglyceride at low dose. Values are expressed as Mean±SEM (n=5). * indicates significant difference when compared to control, at p<0.05.

Tea catechins (Lambert et al., 2007). Vomiting may be associated with gastric damage with very high oral doses to rats lead to 90% lethality associated with haemorrhagic lesions in the stomach and intestines (Isbrucker et al., 2006). Interestingly, animals with lower absorption rates
Table 9. Mean±SEM of the effect of varying doses of aqueous leaf extract of *T. occidentalis* on biochemical parameters in mice.

<table>
<thead>
<tr>
<th>Group</th>
<th>Na (mmol/L)</th>
<th>K (mmol/L)</th>
<th>ALP (mmol/L)</th>
<th>ALT (mmol/L)</th>
<th>AST (mmol/L)</th>
<th>Urea (mmol/l)</th>
<th>Creat. (mmol/l)</th>
<th>Chol. (mmol/l)</th>
<th>Trigly. (mmol/L)</th>
<th>Total protein (mmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>135.80±3.90</td>
<td>7.20±0.13</td>
<td>51.80±2.48</td>
<td>145.20±3.28</td>
<td>228.80±7.53</td>
<td>2.40±0.16</td>
<td>34.00±1.30</td>
<td>1.58±0.11</td>
<td>0.90±0.05</td>
<td>50.60±1.91</td>
</tr>
<tr>
<td>1250 mg/kg</td>
<td>144.20±2.69</td>
<td>6.44±0.15</td>
<td>42.40±2.11</td>
<td>132.20±3.31</td>
<td>204.40±8.93</td>
<td>2.42±0.17</td>
<td>36.00±1.38</td>
<td>1.70±0.05</td>
<td>0.76±0.08</td>
<td>44.80±1.50</td>
</tr>
<tr>
<td>2500 mg/kg</td>
<td>141.00±1.10</td>
<td>6.26±0.11*</td>
<td>42.40±3.78</td>
<td>139.80±5.95</td>
<td>212.20±4.59</td>
<td>2.48±0.18</td>
<td>33.00±1.41</td>
<td>1.68±0.05</td>
<td>0.80±0.03</td>
<td>50.20±3.01</td>
</tr>
<tr>
<td>5000 mg/kg</td>
<td>151.40±5.67*</td>
<td>6.62±0.27</td>
<td>49.80±2.35</td>
<td>137.00±3.33</td>
<td>206.20±4.96</td>
<td>2.64±0.14</td>
<td>35.60±0.75</td>
<td>1.66±0.08</td>
<td>0.82±0.02</td>
<td>43.80±1.16</td>
</tr>
</tbody>
</table>

The parameters were not statistically significant except Na⁺ that was significantly high at high dose and K⁺ that was significantly low at moderate dose. Values are expressed as Mean±SEM (n=5). * indicates significant difference when compared to control, at p< 0.05.

Table 10. Mean±SEM of the effect of varying doses of aqueous leaf extract of *P. nigrescens* on biochemical parameters in mice.

<table>
<thead>
<tr>
<th>Group</th>
<th>Na (mmol/L)</th>
<th>K (mmol/L)</th>
<th>ALP (mmol/L)</th>
<th>ALT (mmol/L)</th>
<th>AST (mmol/L)</th>
<th>UREA (mmol/L)</th>
<th>CREAT. (mmol/L)</th>
<th>CHOL. (mmol/L)</th>
<th>TRIGLY. (mmol/l)</th>
<th>Total protein (mmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>135.80±3.90</td>
<td>7.20±0.13</td>
<td>51.80±2.48</td>
<td>145.20±3.28</td>
<td>228.80±7.53</td>
<td>2.40±0.16</td>
<td>34.00±1.30</td>
<td>1.58±0.11</td>
<td>0.90±0.05</td>
<td>50.60±1.91</td>
</tr>
<tr>
<td>2000 mg/kg</td>
<td>131.80±3.88</td>
<td>6.42±0.13</td>
<td>45.40±1.50</td>
<td>142.20±4.41</td>
<td>193.80±2.06</td>
<td>2.46±0.17</td>
<td>36.80±1.16</td>
<td>1.64±0.08</td>
<td>0.80±0.03</td>
<td>43.20±1.59</td>
</tr>
<tr>
<td>4000 mg/kg</td>
<td>142.20±0.58</td>
<td>6.34±0.16*</td>
<td>37.40±1.94*</td>
<td>145.40±1.69</td>
<td>189.80±4.90</td>
<td>2.44±0.18</td>
<td>34.20±1.83</td>
<td>1.56±0.10</td>
<td>0.82±0.04</td>
<td>50.40±3.50</td>
</tr>
<tr>
<td>8000 mg/kg</td>
<td>124.20±2.75</td>
<td>6.16±0.07*</td>
<td>47.40±1.91</td>
<td>126.80±2.01*</td>
<td>156.60±17.53*</td>
<td>1.76±0.19</td>
<td>34.80±1.32</td>
<td>1.42±0.08</td>
<td>0.58±0.07*</td>
<td>46.80±0.86</td>
</tr>
</tbody>
</table>

The parameters were not statistically significant except significant reduction in K and ALP at moderate dose and K, ALT, AST and triglyceride that was significantly low at high doses. Values are expressed as Mean±SEM (n=5). * indicates significant difference when compared to control, at p< 0.05.

Table 11. Mean±SEM of the effect of aqueous leaves extract of *C. sinensis* on food intake (g), body weight gain (kg) and relative organ weight (g) in mice.

<table>
<thead>
<tr>
<th>Group</th>
<th>Food intake</th>
<th>Body weight</th>
<th>Kidney weight</th>
<th>Liver weight</th>
<th>Stomach weight</th>
<th>Spleen weight</th>
<th>Testis weight</th>
<th>Heart weight</th>
<th>Lung weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>3.33±0.00</td>
<td>23.27±0.56</td>
<td>0.01±0.00</td>
<td>0.04±0.00</td>
<td>0.03±0.00</td>
<td>0.01±0.00</td>
<td>0.00±0.00</td>
<td>0.01±0.00</td>
<td>0.01±0.00</td>
</tr>
<tr>
<td>700 mg/kg</td>
<td>2.95±0.05*</td>
<td>22.88±0.46</td>
<td>0.01±0.00</td>
<td>0.04±0.00</td>
<td>0.02±0.00</td>
<td>0.01±0.00</td>
<td>0.00±0.00</td>
<td>0.01±0.00</td>
<td>0.01±0.00</td>
</tr>
<tr>
<td>1400 mg/kg</td>
<td>2.83±0.06*</td>
<td>22.74±0.24</td>
<td>0.01±0.00</td>
<td>0.04±0.00</td>
<td>0.01±0.00</td>
<td>0.01±0.00</td>
<td>0.00±0.00</td>
<td>0.01±0.00</td>
<td>0.01±0.00</td>
</tr>
<tr>
<td>2800 mg/kg</td>
<td>2.61±0.07*</td>
<td>22.43±0.32</td>
<td>0.01±0.00</td>
<td>0.04±0.00</td>
<td>0.01±0.00</td>
<td>0.01±0.00</td>
<td>0.00±0.00</td>
<td>0.01±0.00</td>
<td>0.01±0.00</td>
</tr>
</tbody>
</table>

The extracts at different dose against all parameters did not show any significant difference when compared with control. However, Food intake at various doses was significantly decreased. Values are expressed as Mean±SEM (n=5). *Indicates significant difference when compared to control at p< 0.05.

of epigallocatechin gallate (EGCG) suffered greater intestinal but less systemic damage (liver and kidneys) (Galati et al., 2006; Lambert et al., 2007). More so, consumption (at moderate doses) of supplemental forms of Green Tea Catechins (*C. sinensis*) have been repeatedly found to be safe (Chow et al., 2003; Ullmann et al., 2003; Ullmann et al., 2004).

In conclusion, the aqueous leaves extracts of *C.*
Figure 4. Comparative effects of *C. sinensis*, *T. occidentalis* and *P. nigrescens* moderate doses on haematologic parameters in mice.

Figure 5. Comparative effects of *C. sinensis*, *T. occidentalis* and *P. nigrescens* moderate doses on biochemical parameters in mice.

*C. sinensis*, *T. occidentalis* and *P. nigrescens* are moderately safe as medicinal plants. This study established that all the three aqueous leaves extracts of these plants showed haemopoietic enhancement potential which is exhibited in a descending order: *T. occidentalis* > *C. sinensis* > *P. nigrescens* (Figures 4 and 5). Further studies to evaluate their potential roles in radiation-counter-effect, and determining their pharmacokinetic profiles in both animal and human subjects are thus, recommended.

**CONFLICT OF INTERESTS**

The authors have not declared any conflict of interests.

**ACKNOWLEDGEMENT**

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samples analysis.

REFERENCES


Dapsone in topical niosomes for treatment of acne vulgaris

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Acne vulgaris is a common inflammatory skin disorder. It affects the pilosebaceous units followed by an overgrowth by Propionibacterium acne bacteria. Dapsone is a sulfone antibiotic with an anti-inflammatory effect, which is considered to be accountable for its effectiveness in the treatment of acne vulgaris. The present study aims to prepare and evaluate the effectiveness of dapsone niosomes for topical application with an objective to control and prolong the release of the drug with improved skin penetration as a novel formulation for healing of mild to moderate acne vulgaris. Niosomes were formulated by thin film hydration method using different ratios of surfactants (various spans 20, 40, 60 and 80) and cholesterol and were investigated with respect to its shape, size, entrapment efficiency, Fourier transform infrared spectroscopy (FTIR) and in vitro release. Fifteen patients with mild to moderate acne vulgaris were selected and treated with dapsone niosomes as a single topical treatment for their acne lesions. Clinical assessment was done before and after 2 and 8 weeks of treatment. Niosomes containing span 60 showed a higher percentage of drug release after 24 h and greater entrapment efficiency as compared to other formulations. The clinical improvement was noticeable after 2 weeks of treatment with highly significant improvement of acne lesions after 8 weeks of treatment (P < 0.001). Dapsone niosomes is a promising topical formulation for safe, tolerable and effective drug delivery system with minimal side effects apart from mild erythema and post-inflammatory hyperpigmentation.

Key words: Dapsone, acne vulgaris, niosomes, topical, clinical application.

INTRODUCTION

Acne vulgaris is a multifactorial inflammatory skin disease commonly affecting adolescents and young adults with...
considerable physical, psychological, and psychosocial burden on those affected (Zaenglein et al., 2016). Acne is an increased sebum production due to the androgenic effect on the pilosebaceous unit leading to a chronic inflammatory disorder, microbial colonization by Propionibacterium acne, changed keratinization and inflammation of hair follicles in various areas of the body including the forehead, cheek, chest, neck, and back (Williams et al., 2012). No typical therapy for acne, however, a proper medical course for improving lesions can be established for most patients. Convenient local applications of remedies including benzoyl peroxide, retinoids, and antibiotics when utilized in combination usually enhance management of mild to moderate acne (Williams et al., 2012). Dapsone, known as diaminodiphenyl sulfone, is an antibiotic routinely applied with rifampicin and clofazimine as a combination for leprosy therapy (Sago and Hall, 2002). It has antimicrobial and anti-inflammatory effects in diseases which are characterized by the accumulation of neutrophils (Paniker and Levine, 2001). Moreover, it has been utilized for treatment of acne, dermatitis herpetiformis, and various other skin cases (Zhu and Stiller, 2001). It is available both topically and by mouth. However, dapsone can potentially cause severe side effects after oral administration, which may lead to the use of steroids or other antibiotics instead of dapsone to avoid its potential systemic toxicity, although these alternative medications are much less effective (Momen et al., 2014). Topical formulations are made to utilize the drug carriers that guarantee a sufficient localization or permeation of the drug through the skin in order to improve the local effect and decrease the systemic effects or to assure an adequate transdermal absorption (Glavas-Dodov et al., 2003). The therapeutic usefulness of a topical medication requires the effective delivery of an optimal concentration of the active pharmaceutical ingredient through the skin for an appropriate duration (Singh Malik et al., 2016). The variability of penetration of the active drug is dependent on the delivery system and the release pattern of the incorporated drug and this is mainly related to the physicochemical properties of the constituents of that delivery system (Weiss, 2011). Vesicular drug delivery systems using liposomes or niosomes have distinct benefits in terms of better entrapment of drugs, better target site specificity, and controlled drug release profile (Asthana et al., 2016). Niosomes were studied as an alternative to liposome for showing some advantages over liposomes in terms of being more stable, nontoxic, and economic due to the low cost of the nonionic surfactant as compared to phospholipids which are susceptible to oxidation (Nasr et al., 2008). Recently, nonionic surfactant vesicles, known as niosomes, interestingly concerned as permeation enhancers of drugs through the skin (Muzzalupo and Tavano, 2015). Several mechanisms were suggested to explain the penetration enhancing ability of niosomes. They are believed to enhance the horny layer features both by decreasing trans-epidermal water loss which enhances the hydration of stratum corneum and causes loosening of its organized structure. They also modify the barrier function of the stratum corneum by improving its softness through substituting lost skin lipids (Manconi et al., 2006; Abdelkader et al., 2014). Additionally, niosomes have been shown to increase the residence time of trapped substances in the epidermis while decreasing the availability of the drug in the systemic circulation which reduces the side effects (Marianecci et al., 2014). Incorporation of surfactants within niosomes may also improve the efficiency of the drug, probably by promoting its uptake by the target cells. The most suitable surfactants for preparation of niosomes are those with alkyl chain length from C12–C18 (Mura et al., 2007; Nasr et al., 2008; Shilakari et al., 2013; Sharma et al., 2015). Niosomes can be prepared by hydration of synthetic nonionic surfactants either with or without cholesterol. The presence of the steroidal molecule (cholesterol) enhances the solidity of the bilayer and its presence in the cell membrane as an important ingredient affects the bilayer fluidity and permeability. The present study was aimed at the formulation and evaluation of dapsone-loaded niosomes for topical application with an objective to control and prolong the release of the drug with improved skin penetration for treatment of acne. Niosomes (empty and drug loaded) were formulated by utilizing various ratios of surfactant (different span grades 20, 40, 60 and 80) and cholesterol by thin film hydration technique. The in vitro properties of the prepared niosomal formulations and their clinical usefulness for treatment of acne vulgaris were evaluated.

MATERIALS AND METHODS

Dapsone was gifted by El-Nile Co., El- Swah square-America - Cairo, Egypt; sorbitan monolaureate (span 20); sorbitan monopalmitate (span 40), sorbitan monostearate (span 60), sorbitan monooleate (span 80) and cholesterol were purchased from Sigma-Aldrich chemical Co. St. Louis MO (USA) Chloroform, methanol and other chemicals were of analytical grade.

Preparation of niosomal formulation

In the present study, niosomal formulations of dapsone were prepared by thin film hydration technique as reported earlier with slight modifications (Balakrishnan et al., 2009) by using 5 mg of dapsone and a specified amount (300 mmol) of lipids (surfactant: cholesterol) at molar ratio of 9:1, 8:2, 7:3, 6:4, 1:1 and utilizing different grades of spans (span 20, span 40, span 60 and span 80) (Table 1). Accurately weighed quantities of surfactants and cholesterol were taken to give the desired ratio and were dissolved in 10 mL of 2: 1 v/v chloroform/methanol mixture in a round bottom flask. Then, accurately weighed amount of drug was added to the solvent. The solvent was evaporated in a rotary evaporator (Stuart RE300). Germany under a vacuum of 20 inches of Hg at a temperature of 60°C at 150 rpm until a smooth, dry lipid film was obtained followed by introducing under high vacuum through
Table 1. Composition of the prepared dapsone niosomal formulations using various grades of non-ionic surfactants (spans) with different molar ratios of surfactant to cholesterol and the calculated total lipid weight in all formulae.

<table>
<thead>
<tr>
<th>Formulation</th>
<th>Molar ratio</th>
<th>Surfactant (g/mol)</th>
<th>Cholesterol g/mol</th>
<th>Total lipid weight (mg)</th>
<th>Equivalent no. of moles (mmol)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>span 20</td>
<td>Mwt:346.00</td>
<td>Mwt: 386.65</td>
<td></td>
<td></td>
</tr>
<tr>
<td>FA1</td>
<td>1:1</td>
<td>52.00</td>
<td>58.00</td>
<td>110.00</td>
<td>300</td>
</tr>
<tr>
<td>FA2</td>
<td>6:4</td>
<td>62.28</td>
<td>46.40</td>
<td>108.68</td>
<td>300</td>
</tr>
<tr>
<td>FA3</td>
<td>7:3</td>
<td>72.66</td>
<td>35.00</td>
<td>107.46</td>
<td>300</td>
</tr>
<tr>
<td>FA4</td>
<td>8:2</td>
<td>83.55</td>
<td>23.20</td>
<td>106.20</td>
<td>300</td>
</tr>
<tr>
<td>FA5</td>
<td>9:1</td>
<td>93.42</td>
<td>11.60</td>
<td>105.02</td>
<td>300</td>
</tr>
<tr>
<td>span 40</td>
<td>Mwt:403.00</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FB1</td>
<td>1:1</td>
<td>60.45</td>
<td>58.00</td>
<td>118.45</td>
<td>300</td>
</tr>
<tr>
<td>FB2</td>
<td>6:4</td>
<td>72.54</td>
<td>46.40</td>
<td>118.94</td>
<td>300</td>
</tr>
<tr>
<td>FB3</td>
<td>7:3</td>
<td>84.63</td>
<td>35.00</td>
<td>119.43</td>
<td>300</td>
</tr>
<tr>
<td>FB4</td>
<td>8:2</td>
<td>96.72</td>
<td>23.20</td>
<td>119.92</td>
<td>300</td>
</tr>
<tr>
<td>FB5</td>
<td>9:1</td>
<td>108.81</td>
<td>11.60</td>
<td>120.41</td>
<td>300</td>
</tr>
<tr>
<td>span 60</td>
<td>Mwt:431.00</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FC1</td>
<td>1:1</td>
<td>64.65</td>
<td>58.00</td>
<td>122.65</td>
<td>300</td>
</tr>
<tr>
<td>FC2</td>
<td>6:4</td>
<td>77.58</td>
<td>46.40</td>
<td>123.98</td>
<td>300</td>
</tr>
<tr>
<td>FC3</td>
<td>7:3</td>
<td>90.50</td>
<td>35.00</td>
<td>125.50</td>
<td>300</td>
</tr>
<tr>
<td>FC4</td>
<td>8:2</td>
<td>103.44</td>
<td>23.20</td>
<td>126.64</td>
<td>300</td>
</tr>
<tr>
<td>FC5</td>
<td>9:1</td>
<td>116.37</td>
<td>11.60</td>
<td>127.97</td>
<td>300</td>
</tr>
<tr>
<td>span 80</td>
<td>Mwt:429.00</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FD1</td>
<td>1:1</td>
<td>64.35</td>
<td>58.00</td>
<td>122.35</td>
<td>300</td>
</tr>
<tr>
<td>FD2</td>
<td>6:4</td>
<td>77.22</td>
<td>46.40</td>
<td>123.62</td>
<td>300</td>
</tr>
<tr>
<td>FD3</td>
<td>7:3</td>
<td>90.10</td>
<td>35.00</td>
<td>125.10</td>
<td>300</td>
</tr>
<tr>
<td>FD4</td>
<td>8:2</td>
<td>102.96</td>
<td>23.20</td>
<td>126.16</td>
<td>300</td>
</tr>
<tr>
<td>FD5</td>
<td>9:1</td>
<td>115.83</td>
<td>11.60</td>
<td>127.43</td>
<td>300</td>
</tr>
</tbody>
</table>

Vacuum pump for at least three hours for removal of residual content of chloroform. Furthermore, the flask was kept in vacuum desiccators overnight for complete removal of chloroform (Mukherjee et al., 2007). Then, the film was hydrated with 10 mL of PBS pH 7.4 for 1 h at 60°C with shaking on a water bath. The niosomal suspension was retained at 2 to 8°C for 24 h.

**Evaluation of niosomal formulation**

Developed niosomal formulations were evaluated with respect to particle size, shape, entrapment efficiency, FT-IR and in vitro drug release profile.

**Entrapment efficiency**

Entrapment efficiencies of niosomal formulations were determined by centrifugation method. For this, 1 mL niosomal suspension was poured into a centrifugation tube and centrifuged by using cooling centrifuged (REMI cooling centrifuge) at 15000 rpm at 4°C for 10 min. The clear fraction was further used for the determination of free drug by using UV/visible spectrophotometer at 298 nm. The entrapment efficiency was calculated using the following formula:

\[
\text{Entrapment efficiency (EE\%) = } \left( \frac{A_t - A_f}{A_t} \right) \times 100
\]

Where \(A_t\) is the concentration of total drug and \(A_f\) is the concentration of the free un-entrapped drug.

**Particle shape and morphology**

Shape and morphology of selected empty niosomal formulations and drug-loaded niosomal formulations prepared using span 60 were determined by scanning electron microscope with different magnifications.

**Particle size measurement**

The mean particle sizes and zeta potentials of selected niosomal formulations prepared from different grades of spans with the same ratio (FA2, FB2, FC2 and FD2) were analyzed by dynamic light scattering using a Zetasizer Nano ZS (Malvern Instruments, UK) at 25°C. The analysis was carried out after proper dilution in 1:100 deionized water to the concentration appropriate for measurement. The results are shown in Table 2.
 Fourier transform infrared spectroscopy (FT-IR) studies

FT-IR studies were performed to detect the interaction between the drug dapsone, the utilized non-ionic surfactant, and cholesterol. FT-IR spectra were carried out using FT-IR Spectrometer® (Perkin Elmer Instruments) by the potassium bromide (KBr) pellet technique. Samples were blended with KBr powder and pressed to form discs by a torque wrench. Each disc was scanned over the range wavelength 400 to 4000 cm⁻¹. The FT-IR spectra of the selected niosomal formula (FC2) with its components, the raw dapsone, span 60 and cholesterol are displayed in Figure 3.

In vitro drug release

Dapsone release profile from niosomal formulations was determined using the dialysis method. An accurately measured volume of dapsone niosomal formulation equivalent to 0.3 mg/mL transferred to an open-ended glass cylinder (10 cm length and 2.5 cm diameter) and that was sealed at its lower end with presoaked cellulose membrane as dialyzing membrane fitted by elastic ends. The glass cylinder was suspended in a beaker containing 50 mL phosphate buffer (pH 6.8) and was kept at 37±1°C and stirred continuously at 50 rpm using magnetic stirrer. The receptor compartment has been replaced by fresh PBS immediately at the time when the unentrapped drug was completely dialyzed. Then, the release study was performed for the entrapped drug. The release experiments were carried out under sink condition and at predetermined time intervals, 2 mL aliquots were withdrawn from each cylinder and replaced with the same volume of fresh PBS that was kept at the same temperature. The drug content was determined spectrophotometrically at λmax 298 nm. The % cumulative drug release vs time was plotted and shown in Figure 4.

Patient study

The present study was conducted on 15 patients suffering from mild to moderate acne vulgaris according to the global acne grading score system (Adityan et al., 2009). Patients were recruited from attendants of the Dermatology Outpatient Clinic, Minia University Hospital. The duration of scars ranged from 1 to 14 years with a mean of 6.63 ± 4.03. Patients enrolled in the study did not receive any treatment for their acne lesions at least 3 months before the study. An informed consent was taken from each volunteer for treatment, photography and clinical follow-up. This study was approved by the Committee for Postgraduate Studies and Research of Faculty of Medicine, Minia University. All volunteers have been subjected to full history taking, general and local examination and photographing of the lesions. Exclusion criteria included pregnancy, lactation, presence of any skin condition that would interfere with the diagnosis or assessment of acne vulgaris, history of hypersensitivity or allergy to dapsone, or methemoglobinemia or subjects with known G6PD (Glucose 6-Phosphate Dehydrogenase) deficiency or congenital or idiopathic methemoglobinemia. Dapsone niosomes were applied as a topical treatment for acne lesions every night for 8 weeks as a thin film over the acne lesions. Clinical evaluation according to the global acne grading system as well as any side effects were reported before, 2 and 8 weeks after treatment.

Statistical evaluation

The collected data had been analyzed and figured using a computer-based program, SPSS software package for statistical analysis (SPSS for Windows, Version 16.0, copyright ©; SPSS Inc., Chicago, IL, USA). The data had been summarized in the form of mean ± SD. The significance of clinical improvement in the same group was assessed using Dependent (paired) T-test. This was interpreted in the form of P value. The value of *P ≤ 0.05 was regarded statistically significant.

RESULTS AND DISCUSSION

Entrapment efficiency percentage (EE%)

Entrapment efficiency of the drug-loaded niosomal formulation was found to be increased with increasing the cholesterol ratio from 10 to 50% whereas entrapment efficiency decreases on further increase in cholesterol ratio from 1 to 1.5 (Figure 2). This might be due to two aspects: First, with increase cholesterol ratio, hydrophobicity, and stability of bilayers vesicles increase and permeability decrease which may causes an effective entrapping of the hydrophobic drug into the bilayers during formation of vesicles. Second, a larger amount of cholesterol may act competitively with the drug for the packing area inside the bilayer thus preventing the drug as the surfactants assembled. Furthermore, comparing the various niosomal formulations containing different grades of spans (span 20, span 40, span 60 and span 80) at different ratios of surfactant to cholesterol, span 60 containing niosomal formulation (FC2) displayed the highest entrapment efficiency as compared to other formulations. This might be due to the fact that span 60 has longest alkyl chain length compared to other span series (Asthana et al., 2016). Briefly, entrapment efficiency of all niosomal formulations with different grades of spans was found in the following order: Span 60 > span 40 > span 80 > span 20.

<table>
<thead>
<tr>
<th>Formulation</th>
<th>Zeta potential (mV)</th>
<th>Particle size (µm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FA2</td>
<td>-11.67</td>
<td>6.32</td>
</tr>
<tr>
<td>FB2</td>
<td>-18.34</td>
<td>4.80</td>
</tr>
<tr>
<td>FC2</td>
<td>-29.97</td>
<td>2.47</td>
</tr>
<tr>
<td>FD2</td>
<td>-22.11</td>
<td>2.25</td>
</tr>
</tbody>
</table>
Particle shape and morphology

Shape and morphology of niosomal formulations were determined by scanning electron microscopy. It was clearly observed that niosomal vesicles FC1 and FC2 are rounded and symmetrical with a smooth surface and the niosomes were in micrometer size range (Figure 1A). Similarly, the drug-loaded niosomes of the same formulations were almost spherical in shape with larger particle size than the empty ones (Figure 1B). However, some aggregations and curds were displayed in case of FC3 and FC4 niosomal formulation.

Particle size

The particle size of the niosomal formulations prepared using different grades of spans was determined by optical microscopy (OPTEC 097876). It was clearly observed that the particle sizes of niosomes decreased consistently.
from span 20 to span 80 and are found in the following order: Span 20 > span 40 > span 60 > span 80. The particle sizes of empty niosomal formulations were reported in the range of 3.89 to 8.32 μm. This might be due to the increase in the hydrophobicity of the surfactant from span 20 to span 80. The decrease in surface free energy with increasing the hydrophobicity of surfactants may be the major attribute of reduction in the particle size of niosomes. A similar pattern in particle size was observed in case of drug loaded niosomal formulations as previously discussed (Sambhakar et al., 2011). Mean particles' size of drug loaded niosomes was found to be greater than the unloaded niosomes at each ratio of drug: cholesterol: surfactant with different grade of spans (20, 40, 60 and 80). Based on the results, FC2 niosomal formulation was chosen for measuring of zeta potential using Zetasizer. Zeta potential of FC2 was -29.97 mV which indicates good stability of the selected formulation.

**FT-IR studies**

Among the reported amphiphilic molecules, spans was used to investigate the encapsulation of dapsone for topical delivery in treatment of acne. FT-IR studies were performed in order to assure the compatibility between the drug, cholesterol and the utilized surfactant which gave the highest EE% (span 60). Niosomes are self-assembled of spans (amphiphiles) in aqueous media resulting in closed bilayer framework. It was reported that construction of niosomes is rarely extemporaneous and includes some kind of energy such as physical stirring or heating (Sharma et al., 2015). The FT-IR spectra of pure dapsone, span 60, cholesterol, and dapsone-loaded niosomes (FC2) were shown in Figure 3. It can be observed that a band at 3300 to 3400 cm⁻¹ corresponding to the stretch of the amine group (N-H), and peaks corresponding to the bending vibration of -NH₂ groups between 1590 and 1550 cm⁻¹. The bands at 1143 and 1180 cm⁻¹ are ascribed to the symmetric and asymmetric vibrations of the sulfone group (-SO₂). The IR spectrum of cholesterol displays two characteristic peaks in the region between 2800 and 3200 cm⁻¹, which are corresponding to C–H stretching vibrations of methyl groups and vibrations of cyclic hydrocarbons. All the previous bands are clearly observed in the spectrum of niosomal formulation at the same wave numbers. It was revealed that there was no major shifting as well as any loss of functional peaks between the spectra of drug, cholesterol, span 60 and dapsone-loaded noisomes. It
suggests that the formulation components; span 60, cholesterol and dapsone do not interact to form any additional chemical entity but remain as mixture (Chatwal and Anand, 2002).

**In vitro drug release**

*In vitro* studies of selected formulations were carried out in PBS pH 6.8 by dialysis technique (Sathali and Rajalakshmi, 2010) on a magnetic stirrer. It was clearly observed from the data as shown in Figure 4 that *in vitro* drug release of niosomes containing span of different series (60 and 80) was sharply increased up to 24 h. Maximum drug release, that is, 96.78%, was reported in case of niosomes containing span 60 as compared to other series of span 80 after 24 h. The observed increase of *in vitro* release of span 60 formulation might be due to the longest alkyl chain length of span 60 and thus possesses highest release profile (Sambhakar et al., 2011). In contrast, span 80 has monounsaturated alkyl chain and thus has lowest release profile compared to span 60.

**Patient study**

The study included 15 patients with acne vulgaris. Nine patients had mild acne lesions (60%), while the remaining 6 patients had moderate acne (40%). Twelve patients were females (80%) and 3 were males (20%), their age ranged from 15 to 25 years with a mean age ± SD of 19.53 ± 3.04 years. The duration of acne ranged from half to 4 years with a mean duration of 1.53 ± 0.92. Inflammatory lesions (papules and pustules) were present in 8 patients (53%), meanwhile, non-inflammatory lesions (comedones) were the main lesions in 7 (47%) of patients. Before treatment, the clinical evaluation revealed a score ranged from 6 to 28 with a mean score of 15.53 ± 6.8. Clinical improvement was noticeable after 2 weeks of treatment with a highly significant decrease of the score to 9.13 ± 3.99 (P <0.001). With continuing treatment up to 8 weeks, the clinical improvement still showed the highly significant decrease of the score with a mean of 7 ± 3.49 when compared to before treatment (P <0.001). Also, a highly significant improvement was observed when comparing mean scores of lesions after 2 and 8 weeks of treatment (P = 0.001) (Figure 5A). The
effect of dapsone niosomal topical application was clear on both non-inflammatory and inflammatory acne vulgaris during the whole duration of the study. In non-inflamatory, there was a highly significant decrease in the severity of lesions from 9.57 ± 2.15 before treatment to 5.86 ± 2.67 (P =0.001) and 4.29 ± 2.63 (P =0.001) after 2 and 8 weeks of treatment, respectively. There was a non-significant difference when comparing lesion after 2 and 8 weeks (P =0.06) (Figure 5B).

Meanwhile, inflammatory acne lesions showed a highly significant decrease in the severity of lesions from 20.75 ± 4.68 before treatment to 12 ± 2.39 (P < 0.001) and 9.38 ± 2.13 (P = 0.001) after 2 and 8 weeks of treatment, respectively. There was a significant difference when comparing lesion after 2 and 8 weeks (P = 0.009) (Figure 5C). Side effects were minimal apart from mild erythema in 2 patients (13%), which resolved after one week of treatment. Post-inflammatory hyperpigmentation was reported in one case (6.5%) (Figure 5D).

Previous data suggested that the topical dapsone 5% gel showed good efficacy for the alleviation of inflammatory and non-inflammatory acne lesions with no hematological side effects accompanied with oral dapsone (Stotland et al., 2009; Kircik, 2010). In the present study, a novel formulation was introduced for dapsone as niosomes which allowed good penetration and absorption of active material with lower risk of either local irritation or systemic absorption. The once-daily topical application of dapsone demonstrated very promising results with obvious clinical improvement as early as 2 weeks of initiation of treatment, which is maintained with better response up to 8 weeks (P < 0.001). Also, a highly significant improvement was observed when comparing the mean scores of lesions after 2 and 8 weeks of treatment (P <0.001). Despite the fact that topical therapy is preferable by both acne patients and the physicians, no topical mediation has essentially provided an anti-inflammatory action (Kircik, 2010). Both inflammatory and non-inflammatory lesions of acne vulgaris responds well to dapsone niosomal topical application with more pronounced effect on the mean score of inflammatory lesions (P < 0.001).

The obtained results were in agreement with previous study (Al-Salama and Deeks, 2017) which reported a reduction in acne severity (as per the Global Acne Assessment Score) and lesion counts with once-daily dapsone 7.5% versus vehicle. The advantages of using dapsone 7.5% gel over vehicle were observed as early as the second week for inflammatory lesion counts, and from the fourth week or the eighth week for other results. In the present study, side effects were minimal apart from mild erythema in 2 patients, which resolved after one week of treatment. Post-inflammatory hyperpigmentation was also reported in one case. Post inflammatory hyperpigmentation is a recurrent problematic issue and may represent the consequence of many cutaneous

Figure 4. Comparative in vitro release profile of selected dapsone-loaded niosomal formulations in phosphate buffer pH 6.8 at 37±1°C.
diseases as well as therapeutic medications resulted from hypersensitivity responses especially with dark skin populations (Chang, 2009). These results reflect the safety and tolerability of the dapsone niosomes when compared with dapsone gel 7.5% which showed adverse effects of mild to moderate severity as stinging/burning, dryness, scaling, and erythema (Thiboutot et al., 2016). In a previous study, dapsone niosomal gel was studied for its efficiency in treatment of cutaneous leishmaniasis (Aflatoonian et al., 2016). They found it as a promising alternative therapy for oral treatment with fewer side effects. However, they did not report any results for physicochemical characteristics of the prepared niosomes including particle size analysis, encapsulation efficiency or in vitro release study. Also, dapsone was incorporated into nanoemulsion which promoted its permeation through the skin (Borges et al., 2013). Nevertheless, to the best of the authors knowledge, there are no reports in the literature discussing the effect of dapsone niosomes on acne vulgaris.

**Conclusion**

The main focus of this study was the formulation and evaluation of dapsone niosomes for clinical behavior. Niosomal formulae (empty and drug loaded) were prepared by utilizing various ratios of surfactant (different span grades 20, 40, 60, and 80) and cholesterol by thin film hydration method and were investigated for their in vitro characteristics including particle size, shape, FT-IR, EE% and in vitro release pattern. Span 60 containing niosomal formulation (FC2) showed the highest entrapment efficiency with satisfied spherically shaped particles and a size of 3.65 μm. Moreover, FC2 showed the highest percentage of prolonged drug release over 24 h as compared to other formulae. This study revealed that niosomal preparations provide sustained and prolonged delivery of drug with enhanced clinical usefulness. It highlights the effect of dapsone niosomes as a topical once-daily treatment of mild to moderate acne vulgaris with early and maintained clinical improvement.

**Figure 5.** (A) Patient with mild acne vulgaris in the right cheek of 15 years-old female (B) Patient with non-inflammatory (comedonal) acne in the forehead of 20 years-old female (C) Patient with inflammatory (papulo-pustular) in the lower face of 23 years-old female. (D) Patient with inflammatory acne vulgaris with residual post-inflammatory hyperpigmentation (All before and after 8 weeks of treatment with dapsone niosomes).
together with minimal side effects. The authors are aware that one of the limitations of the present study is relatively the small number of patients and the cost of niosome preparation. Hence, further studies on a larger group of patients are needed to confirm and clarify such findings.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

REFERENCES


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