African Journal of Pharmacy and Pharmacology

Volume 10 Number 44, 29 November, 2016
ISSN 1996-0816

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Vol. 10(44), pp. 936-944, 29 November, 2016
DOI: 10.5897/AJPP2016.4665
Article Number: 3042DC461981
ISSN 1996-0816
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African Journal of Pharmacy and Pharmacology

Full Length Research Paper

Myelo-protective and haematopoietic effects of seed extract fractions of *Phoenix dactylifera* in Wistar rats

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Received 25 August, 2016; Accepted 5 October, 2016

Myelo-protective and haematopoietic effects of seed extract fractions (SEF) of *Phoenix dactylifera* were investigated in Wistar rats. The acute toxicity of the SEF were determined in mice (n=12). Wistar rats (n=45), aged 2 to 3 months and weighing 150 to 220 g were grouped into 9, labeled (A to I). Groups A to D were intraperitoneally-induced for myelo-suppression with 3 mg/kg bodyweight (b.wt) of cyclophosphamide for 7 days. Groups A to Horally received graded-doses of SEF (A = (SEF1)100, B = (SEF1) 200, C = (SEF2) 100, D = (SEF2) 200, E = (SEF1) 100, F = (SEF1) 200, G = (SEF2) 100, and H = (SEF2) 200 mg/kg b.wt) for 21 days. Group I served as control. Blood samples (2.0 ml) were collected from each rat on days 8 and 15 into tri-potassium ethylene diaminetetraacetic acid anticoagulant containers and analyzed using haematological auto analyzer (Sysmex KX-21N) following manufacturers guideline. Bone marrow was collected from myelo-suppressed groups (B, D) and normal groups (F, H) on days 15 and 22 into fetal calf serum for cell count. The acute toxicity test revealed an oral LD_{50} of 2000 mg/kg b.wt. The SEF revealed flavonoids, saponins, tannin, proteins, reducing sugars and steroids. On day 8, the myelo-suppressed and normal groups revealed dose-dependentnon significant increase ($p > 0.05$) in haemoglobin, haematocrit, RBC and total WBC compared to control. On day 15, the myelo-suppressed and normal groups revealed dose- and time-dependent significant increase ($p<0.05$) in haemoglobin, haematocrit, RBC and total WBC and significant decrease ($p < 0.05$) in bone marrow cells of group B compared to control. Day 22 revealed significant increase ($p < 0.05$) in bone marrow cells of groups B, D, F and H compared to control. The observed effects indicate myelo-protective and haemopoietic potentials of the SEF in Wistar rats.

Key words: *Phoenix dactylifera,* anaemia, graded-doses, myelo-protection, haematopoietic.

INTRODUCTION

Treatment of oncology patients with cytotoxic drugs affect haematopoietic cells, especially the granulocyte-macrophage progenitors (CFU-GM) which results to neutropenia (Ozkan et al., 2005). Neutropenia is a

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decrease in circulating neutrophil in the peripheral blood. It is essential to introduce means to provide myelo-protective effects (Nichols et al., 1994). Biological response modifiers have been synthesized to circumvent this haematopoietic toxicity by the cytotoxic drugs. The colony-stimulating factors and interleukins regulate the proliferation of tumor-killing T lymphocytes and so called natural killer cells by inducing cell viability (Kiss et al., 2004).

Apoptosis of haematopoietic progenitors exposed to DNA-damaging drugs or γ-irradiation is mediated by p53 (Alyasiri et al., 2011). Furthermore, p53 appears to be a key regulator of the proliferation of hematopoietic progenitors, as p53 status influences both long- and short-term repopulation following bone marrow transplantation. Other approaches such as employing herbal medicine with a view to myelo-protection have equally been pursued (Ufelle et al., 2011).

Phoenix dactylifera possess numerous medicinal properties and is used in the treatment of stroke, building up body weight, help in slowing ageing and in treatment of toothache (Biglari et al., 2008). The extract of the date have exhibited anti-diabetic, anti- inflammatory and anti-oxidant activity (Michael et al., 2013; Rahmani et al., 2014).

Most anti-neoplastic agents are known to cause myelo-suppression. It has also been demonstrated that various crude and fractions of *Vitex doniana* leaves extracts have myelo-protective activity in cyclophosphamide-induced myelo-toxicity (Ufelle et al., 2011). There is paucity of data on the myelo-protective and haematopoietic effects of SEF of *Phoenix dactylifera*. The aim of this study was to investigate the myelo-protective effects of SEF of *P. dactylifera* in myelo-suppressed and normal Wistar rats. The specific objectives were to determine the acute toxicity (LD_{50}) of the seed extract in mice, fractionate the extract using column chromatography and Gas Chromatography Mass Spectrometry (GC-MS), haematological parameters and bone marrow count of the myelo-suppressed and normal Wistar rats after oral administration of SEF of *P. dactylifera*.

MATERIALS AND METHODS

Collection of the plant materials

The fruits of *P. dactylifera* were bought from Daji market, Sokoto, Sokoto State, Nigeria. It was authenticated by a taxonomist in the Department of Plant Science and Biotechnology, University of Nigeria Nsukka Campus, Nigeria. A voucher specimen (UNH-M3) was kept in the herbarium unit for future reference.

Animal housing

Wistar rats (n=45) were purchased and housed in the Animal House of College of Medicine, University of Nigeria Enugu Campus. They were allowed to acclimatize for two weeks and fed with commercially available rat feed and have access to water and feed and have access to water and feed *ad libitum*.Wistar rats were handled in this study according to International guidelines for handling experimental animals by American Physiological Society (APS).

Preparation of extract

The seeds (150 g) were harvested from its fruits, shade-dried and grinded into fine power and then soaked in 2.5L of methanol for 48 h. Filtration was carried out using what man Number 1 filter paper. The filtrate was evaporated to dryness. The dried extract(18.75 g) was scrapped out of the stainless bowl giving a percentage yield of 12.5 %. Ten (10)grams of the extract was dissolved in 100 ml of distilled water to get a concentration of 100 mg/ml, ready for use.

Acute toxicity test: (median lethal dose, LD_{50})

This was performed on mice according to the procedure described by Lorke (1983). The LD_{50} was performed in two stages. In the first stage, 3 groups of 3 mice each were treated with 10, 100 and 1000 mg/kg b.wt of the extract and observed for number of deaths in 24 h. Based on the percentage survival rates, 4 mice were treated with 1500, 2000, 2500 and 3000 mg/kg b.wt of extract in the second stage and the number of deaths in 24 h recorded. The LD_{50} was calculated as the geometric mean of the highest non-lethal and the lowest lethal doses.

Column chromatography was performed according to the method described by Still et al. (1978).

Phytochemical analysis

Phytochemical analysis of seed extract fractions of *P. dactylifera* were done in the Department of Pharmacognosy, University of Nigeria, Nsukka, Nigeria with the method described by Ioan (1984). In general tests for the presence or absence of phytochemical compounds using the above methods involve the addition of an appropriate standard chemical agent to the extract in a test-tube and shaken vigorously or gently as the case may be. Gentle heat may sometimes be required.

Gas chromatography-mass spectrometry (GC-MS sample preparation)

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Extract (0.02 g) was dissolved into 10.0 ml of methylene chloride in GC-MS sample vial.
A screw cap and septa (red side facing out) was placed onto the sample vial.
The sample vial was placed into the sample tray provided for GC-MS samples.
The information requested for the sample was printed onto the log in sheet for the sample tray.
GC-MS-QP 2010 PLUS SHIMADZU JAPAN was used to separate the methanol seeds extract of *P. dactylifera* after column chromatography and named the different compounds (Alon and Amirav, 2006).

Experimental design

The acute toxicity of the SEF were determined in mice (n=12). Wistar rats (n=45), aged 2 to 3 months and weighing 150 to 220 g were grouped into 9, labeled (A to I). Groups A to D were intraperitoneally-induced for myelo-suppression with 3 mg/kg bodyweight (b.wt) of cyclophosphamide for 7 days. Groups A to H

Table 1. The phytochemical analysis results of the seed extract fractions(SEF) of *Phoenix dactylifera.*

Constitutents	SEF1	SEF2
Flavonoids	+	++
Saponins	+	+
Tannins	+	+
Proteins	+	++
Reducing sugars	+	+
Steroids	+	+

- = absent; + = present; ++ = moderately present; sef = seed extract fraction.

orally received graded-doses of SEF (A = (SEF1)100, B = (SEF1) 200, C = (SEF2) 100, D = (SEF2) 200, E = (SEF1) 100, F = (SEF1) 200, G = (SEF2) 100, and H = (SEF2) 200 mg/kg b.wt) for 21 days. Group I served as control.

Sample collection

Blood samples (2.0 ml) were collected from each rat on days 8 and 15 into tri-potassium ethylene diaminetetraacetic acid anticoagulant containers for haematological analysis. Bone marrow was collected from myelo-suppressed groups (B, D) and normal groups (F, H) on days 15 and 22 into fetal calf serum for bone marrow cell count.

Haematological analysis

This was analyzed using haematological auto analyzer (Sysmex KX-21N) following manufacturers guideline.

Bone marrow cell count

Principle: The fetal calf serum makes the bone marrow cells non-adhesive and easy for visual count microscopically (Pawar et al., 2006).

Procedure

Bone marrow was harvested from the femur and suspended in RPMI containing 2 ml of10% fetal calf serum (FCS).The bone marrow cell was counted in a haemocytometer using white blood cell dilution buffer (1% glacial acetic acid) in phosphate buffered saline (PBS) and expressed as total live cells per femur.

Statistical analysis

The data were subjected to descriptive statistics in statistical package for social science computer software version 20 using analysis of variance and student's t-test at 95% confidence interval. Probability value of less than 0.05 was considered significant.

RESULTS AND DISCUSSION

P. dactylifera possess numerous medicinal properties and is used in the treatment of many ailments (Onuh et al., 2012). The extract of date has been reported to possess anti-ulcer, hepato-protective, anti-diarrheal effects. Whereas the methanol and aqueous extract of the date have exhibited anti-inflammatory and anti-oxidant activity by significantly increasing the plasma levels of vitamin C, E and A (Zhang et al., 2013). Dates are being consumed in modern cultures for the pleasant flavor, odour, and their biting texture in addition to their use for flavoring foods, beverages and medicine (Al-shahib and Marshall, 2003). The fruit is a natural source of folic acid, an important micronutrient and independent risk factor for cardiovascular disease because of its tannin content; it is used medicinally as a detersive and astringent in intestinal trouble. Due to paucity of data on the myelo-protective and haematopoietic properties of SEF of *P. dactylifera*, this study was designed to investigate the myelo-protective and haematopoietic effects of SEF of *P. dactylifera* in myelo-suppressed and normal Wistar rats.

The acute toxicity test revealed an oral LD_{50} of 2000 mg/kg b.wt in mice. The observed high LD_{50} of the SEF indicate its safety for consumption. The phytochemical analysis of SEF revealed flavonoids, saponins, tannin, proteins, reducing sugars and steroids Table 1. This indicates the pharmacological potentials of SEF of *P. dactylifera.*

On day 8, the myelo-suppressed groups (A and B) that received graded-doses of SEF 1 revealed significant decrease in haemoglobin, haematocrit, RBC and total WBC when compared with the control. However, the myelo-suppressed groups (C and D) that received graded-doses of SEF 2 revealed dose-dependent increases compared to A and B but were lower than the control values Table 2. The progressive increases in the haematological parameters indicate myelo-protective effect which was more pronounced in SEF 2. The more pronounced effects of SEF 2 may be due to the differences in the concentrations and molecular weight to their chemical constituents. The SEF 2 revealed smaller molecular weight compounds than SEF 1. The observed lower values than the control might be due to the effect of cyclophosphamide that was used to induce myelo-suppression. The observed effects may also be attributed to the duration of SEF administration at the stage and time of sample collection which may not be enough to cause significant effects. The normal groups (E to H)

Table 2. The mean ± SD of haematological parameters of Myelo-suppressed and control Wistar rats on day 8 oral administration of graded-doses of SEF1 and SEF2 of *P. dactylifera*.

Groups/ Parameters	A Myelo-suppressed 100 mg/kg b.wt SEF1	B Myelo-suppressed 200 mg/kg b.wt SEF1	C Myelo-suppressed 100 mg/kg b.wt SEF2	D Myelo-suppressed 200 mg/kg b.wt SEF2	I Control
Hb (g/dL)	6.5 ± 2.5*	7.8 ± 0.9*	10.5 ± 0.5	11.8 ± 1.0	11.5 ± 0.9
Hct (L/L)	0.19 ± 0.01*	0.22 ±0.01*	0.31 ± 0.01	0.34 ± 1.5	0.35 ± 0.02
RBC ($\times10^{12}$/L)	2.3 ± 0.27*	2.8 ± 0.32*	3.3 ± 0.54	3.6 ± 0.46	3.4 ± 0.19
MCHC (g/dL)	32.35 ± 1.5	32.50 ± 0.8	30.88 ±2.1	31.71 ± 0.5	32.86±0.3
MCH (Pg)	23.91 ± 2.3	27.86 ± 2.5	31.82 ±2.1	32.78 ± 1.5	33.82±1.7
MCV (FL)	73.91 ± 4.2	85.71 ± 3.7	103.03 ±5.4	94.44 ± 3.2	102.94 ±4.8
TWBC($\times10^{9}$/L)	1.5 ± 0.29*	2.6 ± 0.27*	3.7 ± 0.36	4.1 ± 0.5	4.4±0.1

* $p < 0.05$ (Significant).

Table 3. The mean ± SD of haematological parameters of normal and control Wistar rats on day 8 oral administration of graded-doses of SEF1 and SEF2 of *P. dactylifera.*

Groups/ parameter	E Normal 100 mg/kg b.wt SEF1	F Normal 200 mg/kg b.wt SEF1	G Normal 100 mg/kgb.wt SEF2	H Normal 200 mg/kg b.wt SEF2	I Control
Hb (g/dL)	11.6 ± 2.5	12.1 ± 0.9	12.5 ± 0.5	12.8 ± 1.0	11.5 ± 0.9
Hct (L/L)	0.34 ±0.01	0.36 ± 0.01	0.37 ± 0.01	0.38 ± 1.5	0.35 ± 0.02
RBC ($\times10^{12}$/L)	3.3 ± 0.27	3.8 ± 0.32	3.5 ± 0.54	4.2 ± 0.46	3.4 ± 0.19
MCHC (g/dL)	32.35 ± 1.5	32.50 ± 0.8	30.88 ± 2.1	31.71 ± 0.5	32.86±0.3
MCH (Pg)	23.91 ± 2.3	27.86 ± 2.5	31.82 ± 2.1	32.78 ± 1.5	33.82±1.7
MCV (FL)	73.91 ± 4.2	85.71 ± 3.7	103.03 ± 5.4	94.44 ± 3.2	102.94 ±4.8
TWBC($\times10^{9}$/L)	3.4 ± 0.29	3.7 ± 0.27	4.5 ± 0.36	5.2 ± 0.5	4.4±0.1

revealed progressive increases in haemoglobin, haematocrit, RBC and total WBC which were not significant when compared with the control Table 3. This may also be attributed to the duration of SEF administration at the stage and time of sample collection which may not be enough to cause significant effects.

On day 15, the myelo-suppressed groups (A and B) revealed significant decrease in haemoglobin, haematocrit, RBC and total WBC when compared with the control. This might be that the constituents SEF could not correct the myelo-suppressive actions of the cyclophosphamide at this stage. However, the parameters recorded progressive increases at increasing dosage of SEF and when day 15 was compared with day 8. The observed effects were dose and time-dependent. This indicates myelo-protective action by the extract (Ragab et al, 2013). The haemoglobin, haematocrit, RBC and total WBC of the myelo-suppressed group C that received lower dose of SEF 2 was not significant but increased significantly in myelo-suppressed group D that received higher dose of SEF 2 Table 4.The SEF might have stimulated erythropoietin production for haematopoiesis as well as the immune system for the leucocytosis. Themyelo-protective effect manifests at increased concentration of SEF. The haemoglobin, haematocrit, RBC and total WBC of normal groups (E and F) that received graded-doses of SEF 1 and group G that received lower dose of SEF 2 were not significant but group H that received higher dose of SEF 2 increased significantly when compared with the control Table 5. The observed effects indicate haematopoietic potentials which was more noticeable in SEF 2. This may be due to the higher concentration and smaller molecular weight compounds contents of SEF 2.

On day 15, bone marrow cell count decreased significantly in group B rats that received SEF 1 when compared with the control. This might be due to duration of extract administration. On day 22, groups B, D, F and H revealed significant increase in bone marrow cell count when compared with the control. The bone marrow cell count of rats that received SEF 2 was higher than those that received SEF 1 Table 6. The observed effects indicate myelo-protective and haemopoietic potentials of the SEF. The SEF might be stimulating the liver to

Table 4. The mean ± SD of haematological parameters of myelo-suppressed and control Wistar rats on day 15 oral administration of graded-doses of SEF1 and SEF2 of *P. dactylifera*.

Groups/ parameter	A Myelo-suppressed 100 mg/kg b.wt SEF1	B Myelo-suppressed 200 mg/kg b.wt SEF1	C Myelo-suppressed 100 mg/kg b.wt SEF2	D Myelo-suppressed 200mg/kg b.wt SEF2	I Control
Hb (g/dL)	8.5 ± 1.5*	9.8 ± 0.5*	12.5 ± 0.4	13.8 ± 0.5*	11.5 ± 0.9
Hct (L/L)	0.24± 0.01*	0.28 ±0.01*	0.36 ± 0.01	0.39 ± 1.5*	0.35 ± 0.02
RBC ($\times 10^{12}$/L)	3.2 ± 0.27*	3.7 ± 0.32*	4.1 ± 0.54	4.5 ± 0.46*	3.4 ± 0.19
MCHC (g/dL)	32.35 ± 1.5	32.50 ± 0.8	30.88 ±2.1	31.71 ± 0.5	32.86±0.3
MCH (Pg)	23.91 ± 2.3	27.86 ± 2.5	31.82 ±2.1	32.78 ± 1.5	33.82±1.7
MCV (FL)	73.91 ± 4.2	85.71 ± 3.7	103.03 ±5.4	94.44 ± 3.2	102.94 ±4.8
TWBC($\times 10^{9}$/L)	2.4 ± 0.29*	3.5 ± 0.27*	4.6 ± 0.36	5.0 ± 0.5*	4.4±0.1

*$p < 0.05$ (Significant).

Table 5. The mean ± SD of haematological parameters of normal and control Wistar rats on day 15 oral administration of graded-doses of SEF1 and SEF2 of *P. dactylifera*.

Groups/ parameter	E Normal 100 mg/kg b.wt SEF1	F Normal 200 mg/kg b.wt SEF1	G Normal 100 mg/kg b.wt SEF2	H Normal 200 mg/kg b.wt SEF2	I Control
Hb (g/dL)	11.9 ± 2.5	12.6 ± 0.9	12.8 ± 0.5	13.3 ± 1.0*	11.5 ± 0.9
Hct (L/L)	0.34 ±0.01	0.36 ± 0.01	0.37 ± 0.01	0.39 ± 1.5*	0.35 ± 0.02
RBC ($x10^{12}$/L)	3.3 ± 0.27	3.8 ± 0.32	3.4 ± 0.54	4.1 ± 0.46*	3.4 ± 0.19
MCHC (g/dL)	32.35 ± 1.5	32.50 ± 0.8	30.88 ± 2.1	31.71 ± 0.5	32.86±0.3
MCH (Pg)	23.91 ± 2.3	27.86 ± 2.5	31.82 ± 2.1	32.78 ± 1.5	33.82±1.7
MCV (FL)	73.91 ± 4.2	85.71 ± 3.7	103.03 ± 5.4	94.44 ± 3.2	102.94 ±4.8
TWBC($x10^{9}$/L)	3.5 ± 0.29	3.6 ± 0.27	4.7 ± 0.36	5.1 ± 0.5*	4.4±0.1

Table 6. The mean ± SD ofBone Marrow cell count of myelo-suppressed and normal rats on days 15 and 22 of the study.

Groups/ cellularity	B Myelo-suppressed 200 mg/kg b.wt SEF1	D Myelo-suppressed 200 mg/kg b.wt SEF2	F Normal 200 mg/kg b.wt SEF1	H Normal 200 mg/kg b.wt SEF2	Control
Day 15 ($\times 10^{6}$/femur)	7.80±0.77*	10.30± 1.5	8.40 ± 0.5	11.62 ± 1.2	10.25± 1.3
Day 22 ($\times 10^{6}$/femur)	12.37 ±0.65*	13.50 ± 1.5*	12.72±0.9*	13.85 ± 0.48*	

* $p < 0.05$ (Significant)

synthesize more erythropoietin to cause haematopoiesis. The observed effects were more consistent in SEF2 probably due to the smaller molecular weight compounds in SEF2 which may have easier penetration into the tissue to cause the observed effects. The GC-MS of SEF 1 and SEF 2 of Phoenix datiliferawith their Formulae, molecular weights and compound names are shown in Figures 1 to 12.

In conclusion, this study has demonstrated myelo-protective and haematopoietic properties by SEF as shown by the observed progressive increases in the parameters of both the myelo-suppressed and normal Wistar rats.

Conflicts of Interests

The authors have not declared any conflict of interests.

ACKNOWLEDGEMENTS

Authors wish to acknowledge the taxonomist from the Department of Plant Science and Biotechnology, University of Nigeria Nsukka Campus, Nigeria.

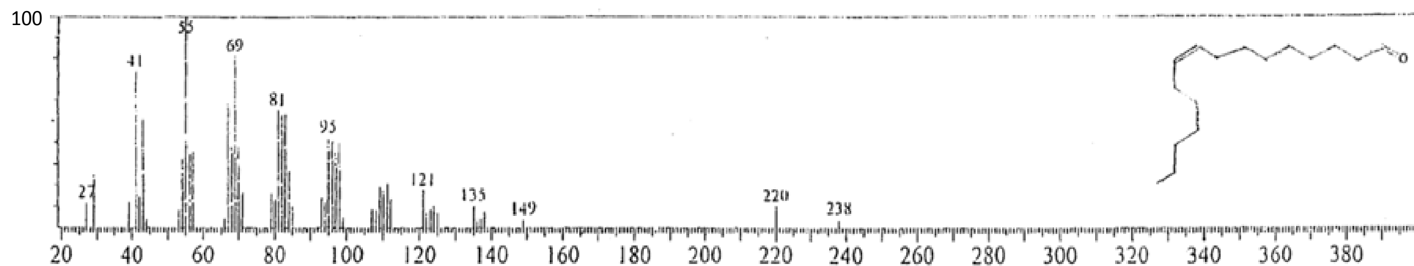

Figure 1. SEF1. Formula: C57H 10406; CAS: 537-39-3; Molecular weight: 884; compound name: 9-Octadecenoic acid, I, 2, 3-propanetriyl ester, (E, E, E) - $$ 2, 3-Bis [(9E)-9-octadecenoyloxy) propyl (9E)-9-octadecenoate # $.

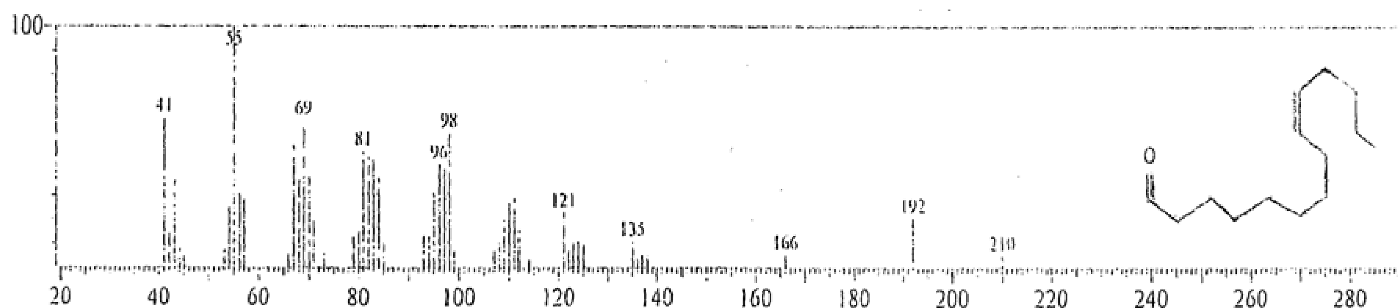

Figure 2. SEF1. Formula: C I 6H300; CAS: 562 19-04-6; Molecular Weight: 238; Compound Name: cis-9-Hexadecenal $$ 9-1-lexadecenal, (Z) - $ (Z)-9-Hexadecenal $ Z-9-Hexadecenal $$ (9Z)-9-Hexadecenal # $.

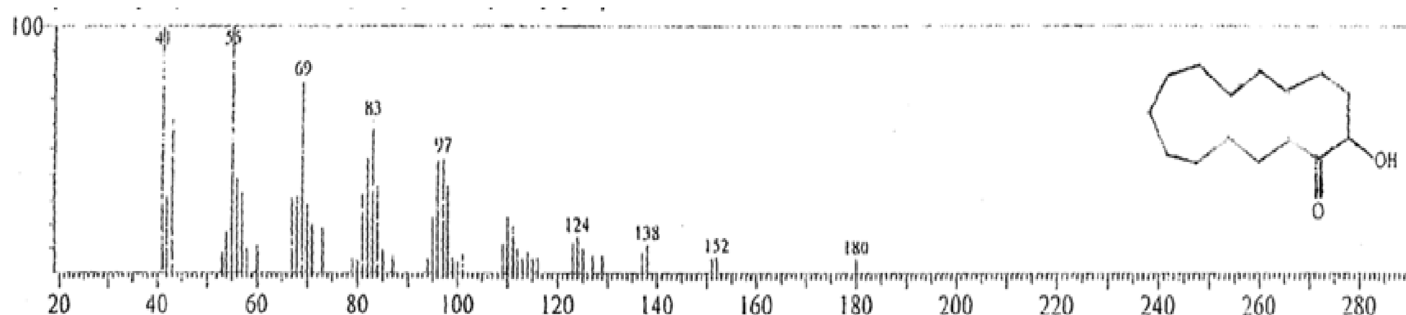

Figure 3. SEF1. Formula: C1 8H3402; CAS: 112-80-1; Molecular Weight: 282; Compound Name: Oleic Acid S 9-Octadecenoic acid (Z) - $$ delta (Sup9)-cis-Oleic acid $$ cis-.dJta. (Sup9)-Octadecenoic acid S$ cis-Oleic Acid $$ cis-9-Octad.

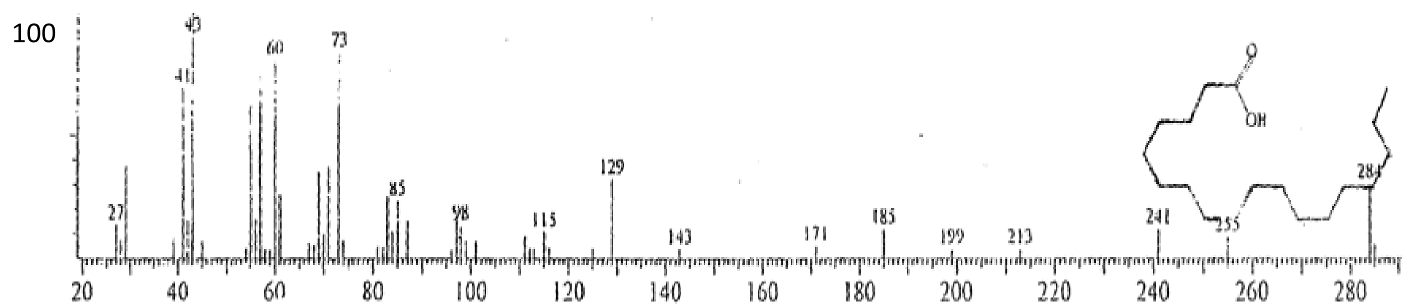

Figure 4. SEF1. Formula: C I 7H320; CAS: 6O6O953-2; Molecular Weight: 252; Compound Name: 8-Hexadecenal, 14-methyl-, (Z)- $ 14-Methyl-8-hexadecenal Z (8Z)-14-Methyl-8-hexadecenal # S$

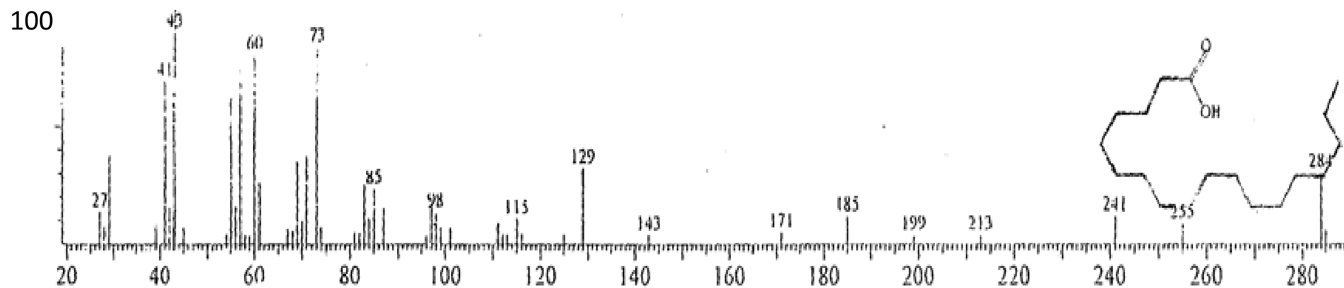

Figure 5. SEF1. Formula: CI 8H3602; CAS: 57- I 4; Molecular Weight: 284; Compound Name: Octadecanioc acid SS Stearic acid n-Octadecanoic acid Humko Industrene R SS Hydrofol Acid 150 SS Hystrene S-97Hysrene T-70.

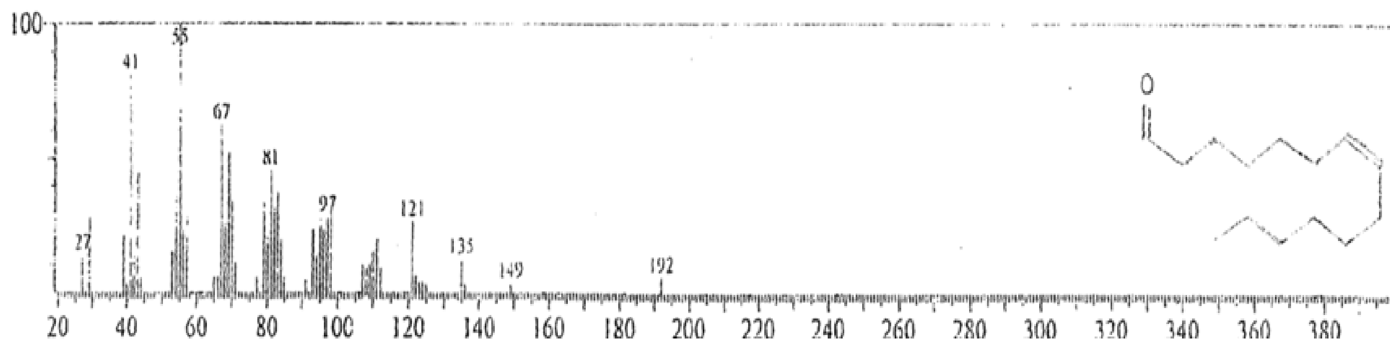

Figure 6. SEF1. Formula: C18H3602; CAS: 57-I -4; Molecular Weight: 284; Compound Name: Octadecanoic acid $$ Stearic acid $S n-Octadecanoic acid $$ Humko 1ndustrene R $$ Hydrofol Acid 50 S$ Hystiene 5-97 $$ Hystrene T-7.

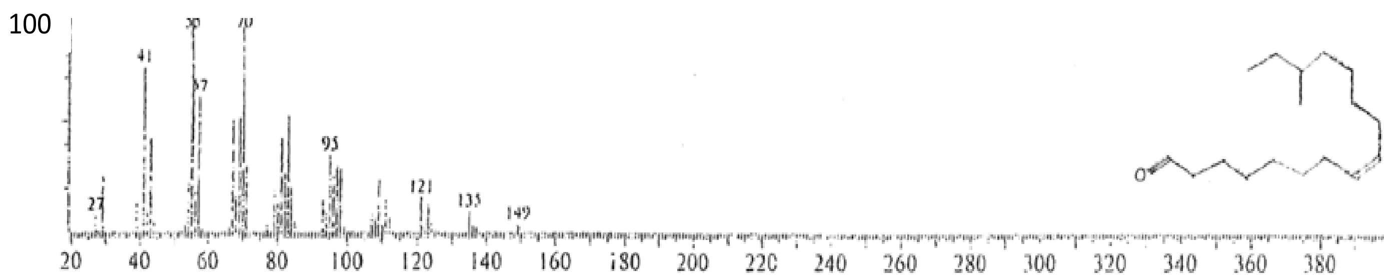

Figure 7. SEF2. Formula: C I 4H260; CAS: 53939-27-8; Molecular Weight: 210; Compound Name: 9-Tetradeceiial, (Z) - S$ (Z)-9-Tetradecenal $$ Z-9-Tetradecenal $$ Z-9-Teadecenol $$ (9Z)-9-Tetradecenal # Z-9-Tetadecenal $$.

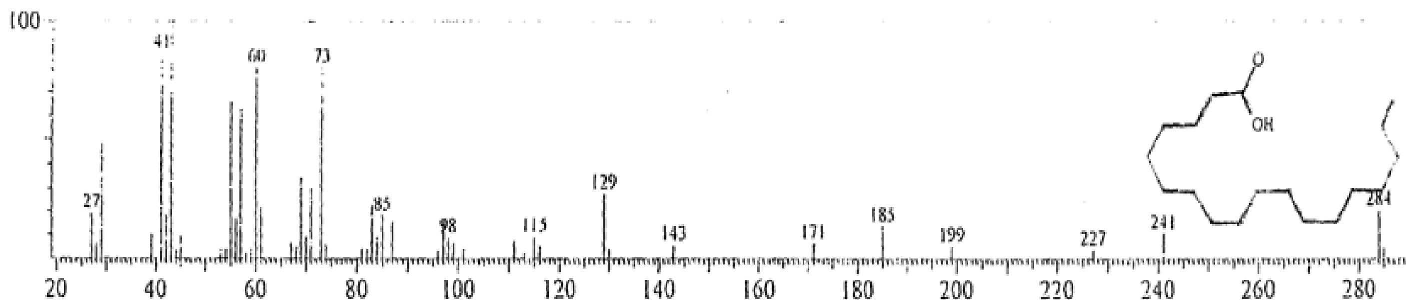

Figure 8. SEF2. Formula: C' I 6H300; CAS: 562I 9-04-6; Molecular Weight: 238; Compound Name: cis-9-Hexadecenal S 9-Hexadecenal, (Z) - $$ (Z)-9-Hexadecen S$ Z-9-Hexadecenal S (9Z)-9-Hexadecenal.

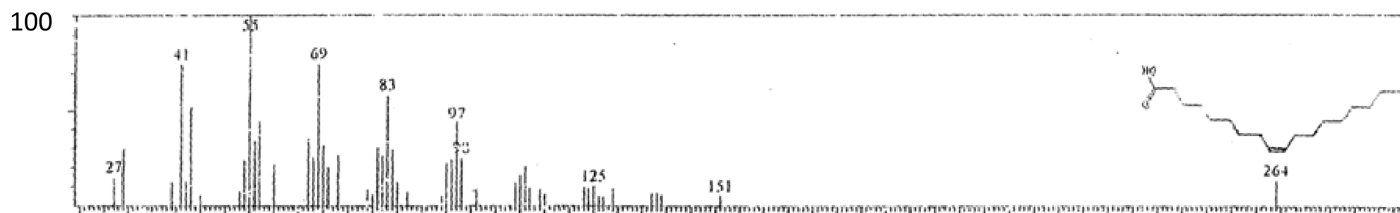

Figure 9. SEF2. Formula: C15H28O2; CAS: 4727-18-8; Molecular Weight: 240; Compound Name: Cyclopentadecanone, 2-hydroxy- $S 2 Hydroxycyclopentadecanone # $$.

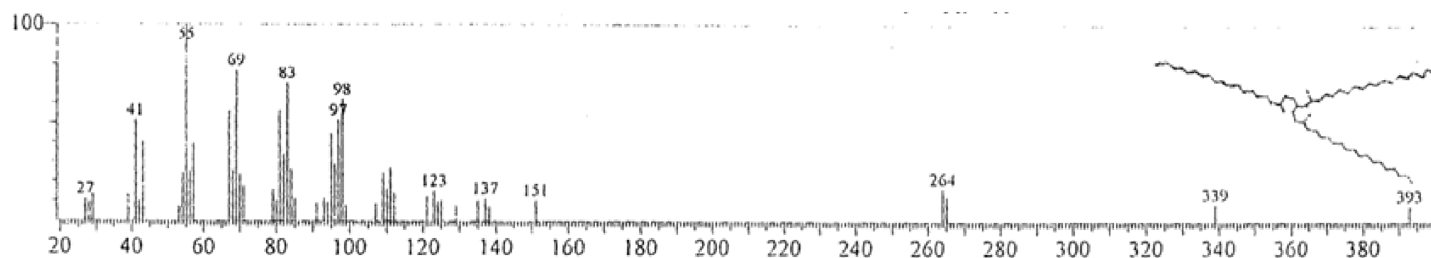

Figure 10. SEF2. Formula: C 8H3602; CAS: 57- 1-4; Molecular weight: 284; Compound Name: Octadecanoic acid S$ Stearic acid n-Octadecanoic acid S$ Humko lndustrene R S Hydrolbi Acid ISO $ Hysirene S-07 Hystrene T-7O.

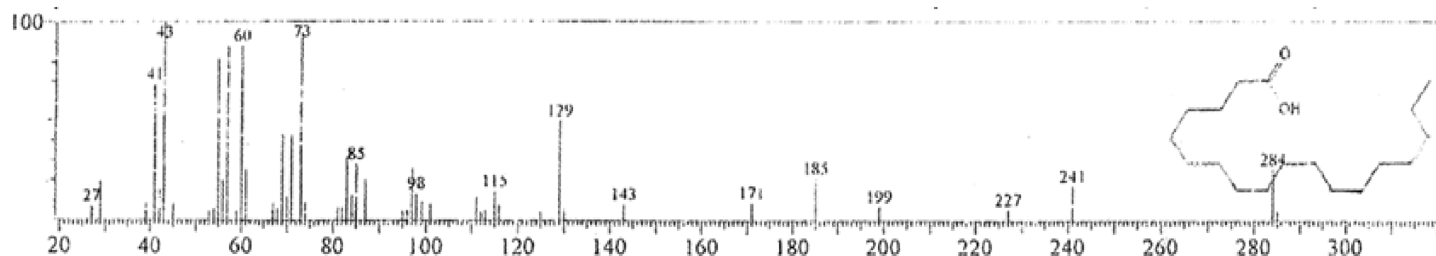

Figure 11. SEF2. Formula: Cl 6H300; CAS: 562 19-04-6; Molecular Weight: 238; Compound Name: cis-9-Hexadecena S$ 9-Hexadecetial, (Z) - $S (Z)-9-Hexadecenal $$ Z-9-Hexadecenal $$ (9Z)-9-Hexadecenal # $$.

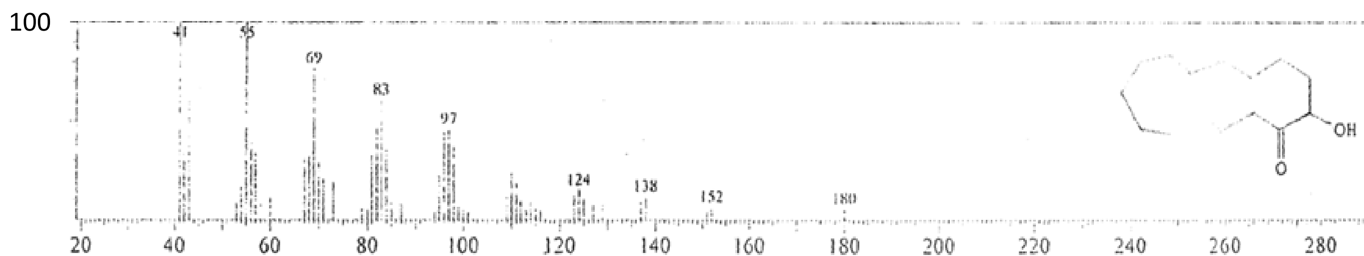

Figure 12. SEF2. Formula: C16H300; CAS: 56219-04-6; Molecular Weight: 238; Compound Name: cis-9-Hexadecenal S$ 9-Hexadecena [(Z) - $$ (Z)-9-Hexadecenai $$ Z-9-Hexadecenal $$ (9Z)-9-Headecenal # $$.

Abbreviations

SD, Standard deviation; **SEF,** seed extract fraction; **b.wt,** body weight; **Hb,** haemoglobin; **Hct,** haematocrit; **RBC,** red blood cell; **MCHC,** mean cell haemoglobin concentration; **MCH,** mean cell haemoglobin; **MCV,** mean

cell volume; **TWBC,** total white blood cell

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Vol. 10(44), pp. 945-953, 29 November, 2016
DOI: 10.5897/AJPP2015.4520
Article Number: D88AB1061983
ISSN 1996-0816
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African Journal of Pharmacy and Pharmacology

Full Length Research Paper

Evaluation of *in vitro* antioxidant, reducing, lipoxygenase and ACE inhibition activity of polyherbal drug linkus

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Received 29 December, 2015; Accepted 18 February, 2016

Oxidative, reductive, lipoxygenase and angiotensin converting enzyme (ACE) activities are the condition where there is an inequity among concentrations can cause a multiple pathological effects. Vast results of medical plants and the remarkable contribution on humans has been observed since centuries. The current study has showed the antioxidant, lipoxygenase, ACE inhibition, urease activity and reducing ability *in vitro* on linkus formulation, including lozenges and syrup. The anti-oxidant activities was assessed by scavenging ability of the linkus on free radical (DPPH; $C_{18}H_{12}N_5O_6$) 2,2'-diphenyl-1-picryl hydrazyl. For lipoxygenase measurements, purified lipoxygenase with lionoleic acid as substrate was used for the activity, however for lipoxygenase activity the thiocholine and diothiobisnitrobenzoic acid (DTNB) was used. Indophenol method has been used for determination of urease activity, however the reducing ability has been assessed by the conversion of ferric into ferrous state. *In vitro* results of linkus syrup as compared to standard showed good anti-oxidant and reducing ability. Moderate activity of urease, lipoxygenase and ACE inhibition were observed with comparison of standard. These activities of polyherbal formulation might be helpful for reducing cough and related symptoms.

Key words: Antioxidant activity, reducing ability, urease activity, lipoxygenase and ACE inhibition, linkus.

INTRODUCTION

Reactive oxygen species (ROS) is the byproduct of oxidative stress under the physiological conditions. These extreme ROS accretion will lead to cell damage, such as damage to proteins, DNA, and lipid membranes. The cell injury/damage is initiated by ROS and has been associated with the development of numerous disease

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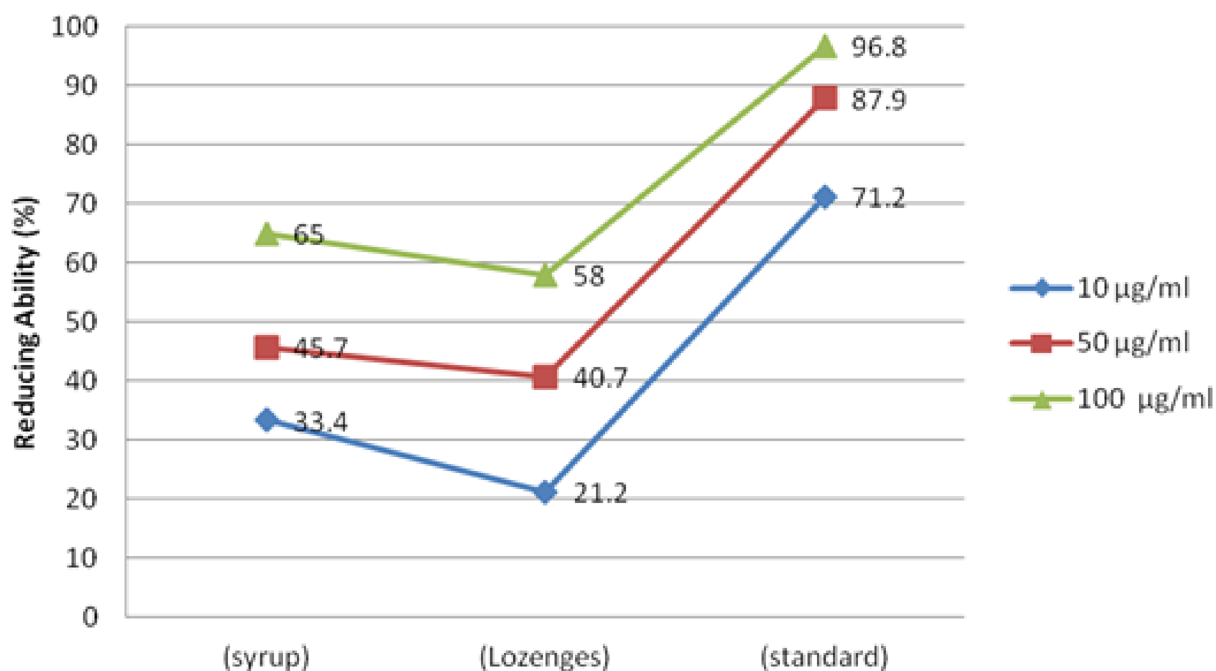

Figure 1. *In vitro* antioxidant activity of LInkus formulation including lozenges and syrup w.r.t standard.

injury/damage is initiated by ROS and has been associated with the development of numerous disease conditions, such as diabetes, atherosclerosis, cancer and cardiovascular disease etc. Its comprising multiple free radicals and exogenous factors play vital role (Finkel et al., 2000).

ROS *in vivo* inside the cell membrane performs multiple mechanism by sunlight or by different chemical and metabolic process, including DNA damage, carcinogenesis etc (Gyamfi et al., 1999; Ganapathy et al., 2011; Gutteridge and Halliwell, 2000; Halliwell, 2001). The free radical, chemicals and toxins creates effects in immune system and are declared as the major contributor of free radicals in the oxidation process (Halliwell, 1994; Kuhnan, 1976; Kumpulainen and Salonen, 1999; Younes, 1981).

Urease is the prominent agent for gastrointestinal track (GI) and help to inhibit *Helicobacter pylori*. Urease also acts directly as virulence factor in infections other than GI, including urinary tract both in humans and animals (Ghous et al., 2010; Halliwell et al., 2008; Nabati et al., 2012). Lipoxygenase is the significant main enzyme for biosynthesis of leukotriene and different potential drug to cope with asthma, arthritis, circulatory diseases etc (Wasserman et al., 1991) (incorporated inside text). Consequently, over a decade, the foremost effort has invested and many vitro active like 5-lipoxygenase inhibitors has developed (Ford-Hutchinson, 1991; Batt, 1992; McMillan and Walker, 1992; Ford-Hutchinson et al., 1994). ACE inhibitors are known to induce dry cough. There has an observation that prostaglandins with others are responsible for this effects but the statement is still controversial (Morice et al., 1987; Gilchrist et al., 1989; Fox et al., 1996).

Naturally occurring antioxidants have effective pharmacological action, including less toxicity and price effectiveness. Multiple plant products, including terpines and phenols also have this activity too (De Souza et al., 2007; Lin and Yin, 2007; Rice-Evans et al., 1996). This present study was based on linkus syrup and lozenges which have a wonder blend of polyherbs, including *Glycyrrhiza glabra, Adhatoda vasica, Viola odorata, Piper longum, Hyssopus officinalis* and *Alpinia galangal* (Appendix 1 and 2). The study focused on anti-oxidant and reducing ability on linkus dosage forms and proved the strength of activity inside the herbal formulation (Figure 1). By proving the activities, it claims that linkus might work on associated symptoms of cough and respiratory tract.

METHODOLOGY

Plant material and techniques

Herb extracts and mentioned chemical constituents were the part of Linkus lozenges and Syrup (Figure 2). Major specification included organoleptic evaluation, qualitative reaction of glycyrrhizic acid, tanning agents and ascorbic acid. Quantitative determination with spectrophotometric evaluation was observed. The total flavonoids contents as luteolin-7-glucoside were not less than 0.080 mg/lozenge (Zeeshan et al., 2014).

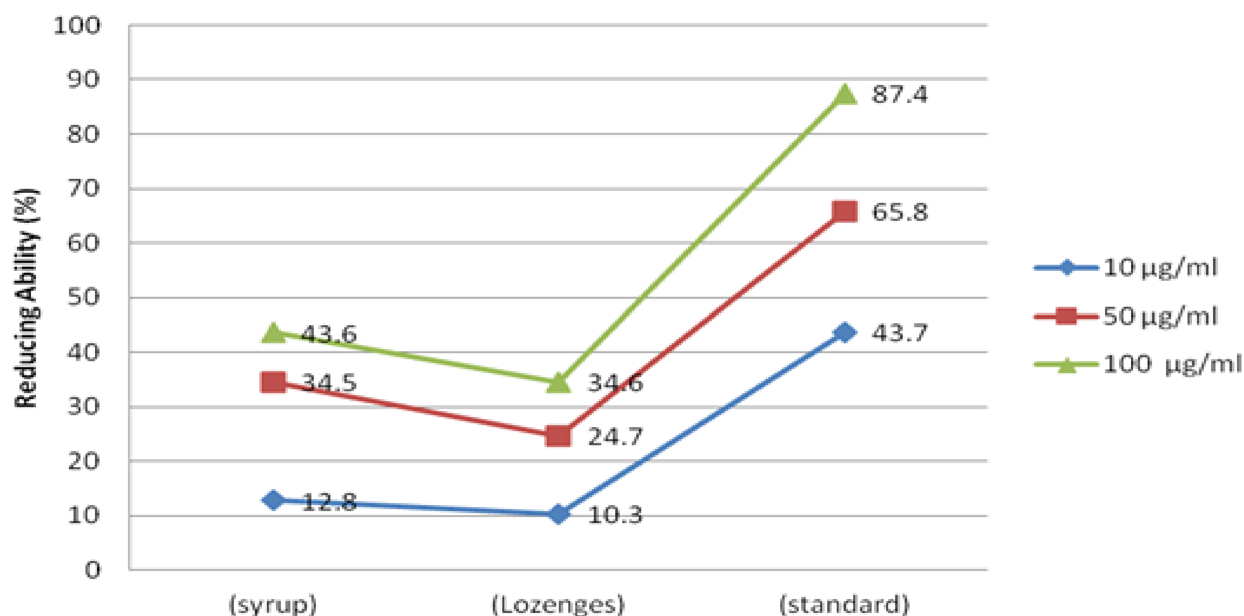

Figure 2. Linkus lozenges and syrup reducing ability w.r.t standard.

Preparation of plant extract

Individual herbs were taken separately, cleaned, grind, weighed and distill water added together with sugar and liquid glucose. Ingredients were transferred from weighing tank to storing tank with temperature range from 110 to 120°C for syrup and 60 to 80°C for lozenges. For lozenges, boiling and vacuum cooking was needed. Kneading, roping and sizing were the next step with the help of uni-plast machine. Lozenges were finally passed through cooling tunnel to obtain the desired hardness. For syrup, hot water filtrate was evaporated via a condenser. Methyl paraben, and Propyl paraben together with flavoring agents were added after completion (Zeeshan et al., 2014)

Chemicals and Reagents

All chemicals were high performance liquid chromatography (HPLC) grade. For reduction, 1, 1-diphenyl-2-picrylhydrazyl was used and obtained from Merck, Pakistan, 2,2'-diphenyl-1-picryl hydrazyl (DPPH) was obtained from Sigma-Aldrich Chemie (Buchs, Switzerland) and used for anti-oxidant activity.

Scavenging activity by DPPH radical

The antioxidant activity was measured by the scavenging aptitude of the syrup and capsules on free radical (DPPH). Antiradical activity analyzed depended on the reduction of DPPH. Its free radicals showed strong absorption at 517 nm due to odd electrons. When this electron was paired in the company of hydrogen donor, for example any antioxidant, the absorption strength decreased and color changed from purple to yellow, with respect to the number of electrons captured (Gülçin et al., 2005). For performing the process, 2, 2-Diphenyl-1-(2, 4, 6-trinitrophenyl) hydrazyl (M.W = 394.24) (Sigma) was prepared in ethanol in a concentration of 3 mM. Each well in 96-well plate was labelled as control, blank and test compound of various concentrations. DPPH solution (95 µl) was added in the labeled wells. The test compound (5 µl) of concentration 10 to 1000 µM in dimethyl sulphoxide (DMSO) was then added in DPPH solution and reaction mixture was mixed for few seconds. The reaction took place in wells when 96 well plates were incubated at 37°C for 30 min. The micro titre plate was read at the absorbance of 515 nm (Spectramax plus 384 Molecular Device, USA) after 30 min. The percentage of radical scavenging activity was considered with respect to DMSO treated control. Butylated hydroxyanisole (BHA) was taken as standard. The DPPH activity was performed with the help of the following equation:

DPPH radical scavenging effect (%) = Ac – As / Ac × 100"

Where As = absorbance of test compound, Ac = absorbance of control

Reducing ability by the conversion of ferric into ferrous state

The reducing ability was determined by the conversion of ferric into ferrous state by antioxidant compounds using the method of Oyaizu (1986). Each test compound (100 µl: 10 to 1000 µM) prepared in DMSO was mixed with phosphate buffer (250 µl: pH 6.6: 0.2 M). Potassium ferricyanide (250 µl: 1%) was then added to the contents in the test tube. This mixture was then incubated at 50°C for twenty minutes in water bath and was centrifuged for ten minutes at 3000 rpm. Subsequently on centrifugation, the top layer of solute (250 µl) was separated in another set of test tubes and mixed with equal volume of DMSO (250 µl). Ferric chloride (0.1 %: 50 µl) was added to the mixture with absorbance at 700 nm on spectrophotometer (Specord 2000, Germany). Percent reduction ability was determined in terms of percentage with respect to BHA used as standard.

Percent reduction activity = At/As × 100

Where As = absorbance of standard, At = absorbance of test.

Table 1. *In-vitro* antioxidant activity of Linkus formulation including lozenges and Syrup w.r.t. Standard.

S\N	Concentration Tested (µg/ml)	Percent Activity (%) (syrup) ± SEM	Percent activity ± SEM (%) (Lozenges)	Percent activity ± SEM (%) (standard)
1	10	33.4±0.9464	21.2±0.421	71.2± 0.41
2	50	45.7 ± 0.6454	40.7 ± 0.443	87.9± 0.45
3		65.0±0.9124	58.0±0.512	96.8± 0.51

Table 2. Linkus lozenges and syrup reducing ability w.r.t. Standard.

S\N	Concentration tested (µg/ml)	Percent Activity (%)(syrup) ± SEM	Percent Activity (%)(capsules)) ± SEM	Percent Activity (%) (standard) ± SEM
1	10	12.8 ± 0.312	10.3 ± 0.131	43.7 ± 0.421
2	50	34.5 ±0.412	24.7± 0.213	65.8 ± 0.321
3	100	43.6± 0.561	34.6 ± 0.312	87.4 ± 0.112

Antiulcer/anti urease activity

By using the indophenol method, the urease activity was evaluated by ammonia production as described by Weatherburn. Reaction mixtures encompassing 25 µl of enzymatic (Jack bean Urease) solution and 55 µl of buffers comprising 100 mM urea were incubated with 5 µl of test compounds for 15 min at 30°C in 96-well plates (Tariq et al., 2011). With the help of indophenol method, activity was determined by measuring ammonia production. After that, 45 µl of phenol reagent (1% w/v phenol and 0.005% w/v sodium nitroprusside) and 70 µl of alkali reagent (0.5% w/v NaOH and 0.1% active chloride NaOCl) were added with each well. By using a microplate reader (Molecular Device, USA), the absorbance at 630 nm was measured after 50 min. In a final volume of 200 µl, all reactions were performed in triplicate. By using SoftMax Pro software (Molecular Device, USA), the results (change in absorbance per min) were processed. All the assays were performed (0.01 M $K_2HPO_4.3H_2O$, 1 mM EDTA and 0.01 M $LiCl_2$) at pH 8.2. Percentage inhibitions were intended from the formula 100 - ($OD_{testwell}$ / $OD_{control}$) × 100. As the standard inhibitor of urease and Thiourea was used (Khan et al., 2013).

Lipoxygenase inhibition activity

Lipoxygenases are family of iron encompassing dioxygenases that convert the addition of molecular oxygen to fatty acid comprising a cis-1, 4- pentadiene classification. The prime product of this response is a "4-hydroperoxycis trans-1, 3-conjugated pentadienyl moiety" within unsaturated fatty acid. This assay processes the hydroperoxides produced in the lipoxygenation reaction using a purified lipoxygenase with lionoleic acid as substrate (Tappel, 1986: Chedea et al., 2012). In the proposed method, lipoxygenase enzyme solution was prepared in sodium phosphate buffer with such concentration to give 130 U per well. Sodium phosphate buffer (pH 8.0: 160 µl:100 mM) was occupied in each well of plate labelled as Blank named B *substrate* and B *enzyme*, as control and Test. Test compound solution in methanol (10 to 1000 µM: 10 µl) was added in each well labelled as test. Lipoxygenase solution (LOX: 20 µl) was added in each well including B *enzyme,* Control and Test except B *substrate* and the mixture was incubated at 25°C for ten minutes. Substrate solution was prepared by adding linoleic acid (155 µl:0.5 mM) into 0.12% w/v tween 20 (257 µl). The mixture was mixed and 0.6 ml NaOH (1 N) was added to remove turbidity and volume was made up to 20 ml with deionized water. This mixture was dispersed with the nitrogen gas to evade autoxidation before adding to each other. The response was started by the adding of 10 µl substrate in each well except enzyme B, also the absorbance was measured at 234 nm for 5 min.

RESULTS

Linkus is the poly herbal formulation analyzed for antioxidant, reducing, and lipoxygenase and ACE inhibition activity with different concentration (10, 50, 100 µl/ml) on 2 dosage of different dosage forms, comprising lozenges and syrup. When formulations of syrup and lozenges were compared at various concentrations (10, 50,100 µg/ml), DPPH radical scavenging activity increased in a dose dependent manner for both formulations just like standard BHA as shown in Table 1. It showed that both dosage forms, including syrup and lozenges have good antioxidant potential that is, 23.4, 45.7, 65.0% w.r.t standard BHA. For determining the reducing activity, ferrous were the leading component. Both dosage forms of syrup and lozenges had some reducing ability as compared to standard as shown in Table 2. For protecting the gastric mucosa, the syrup and lozenges have some anti-urease activity too as shown in Figure 3 and Table 3. Lipoxygenase compounds are the derivatives of arachadonic acid. After analysis, it was determined that the lozenges and capsules have some efficacy for the reduction of inflammation. Results are shown in Table 4.

DISCUSSION

Oxidant cause damage to proteins, macromolecules and DNA and this causes many damages in human tissues

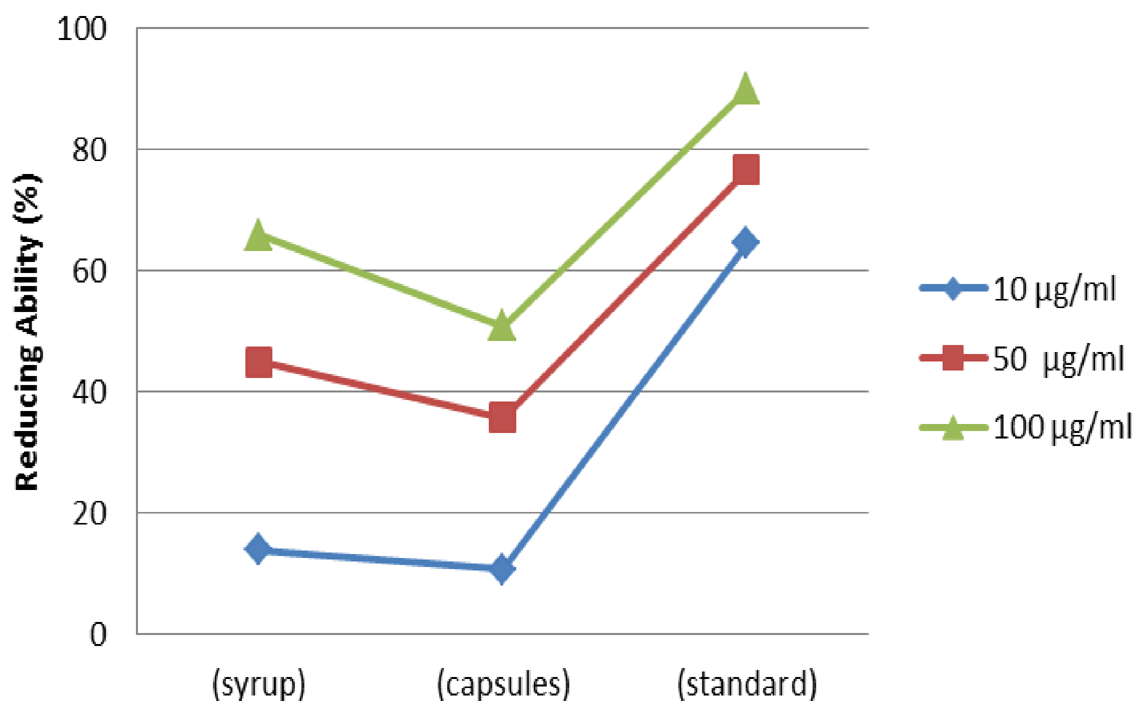

Figure 3. Antiurease activity of linkus lozenges and syrup w.r.t. standard.

Table 3. Antiurease activity of Linkus lozenges and syrup w.r.t. Standard.

S\N	Concentration tested (µg/ml)	Percent activity (%) (syrup)±SEM	Percent activity (%)(capsules)±SEM	Percent activity (%)(standard)±SEM
1	10	13.9±0.121	10.8±0.210	64.5±0.321
2	50	44.9±0.321	35.7±0.412	76.5±0.213
3	100	65.9±0.213	50.8±0.312	89.9±0.312

Table 4. Lipoxygenase inhibiting activity by poly herbal formulation linkus.

S\N	Concentration tested (µg/ml)	Percent Activity (%)(syrup) ± SEM	Percent Activity (%) (capsules) ± SEM	Percent Activity (%)(standard)) ±SEM
1	10	14.9 ± 0.213	12.2 ± 0.312	64.5± 0.410
2	50	46.8 ±0.611	31.0± 0.410	76.5± 0.612
3	100	56.1± 0.412	40.8 ± 0.312	89.9 ± 0.712

including aging (Ames et al., 1992; Fraga et al., 1990; Harman, 1981; Sai et al., 1992; Stadtman et al., 1992; Harman, 1992). Currently, available antioxidant compound including butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) have negative impact on human health (Barlow, 1990; Branen, 1975). For reducing the impact of oxidants, the natural occurring plants have been used for medical purpose (Schuler, 1990). Various plant species have been explored for antioxidant activity (Chu et al.,, 2000; Koleva et al., 2002; Mantle et al., 2000; Oke and Hamburger, 2002). DPPH is the sensitive method for antioxidant screening for plant extracts (Koleva et al., 2002). For determining the antioxidant activity in poly herbal formulation, scavenging

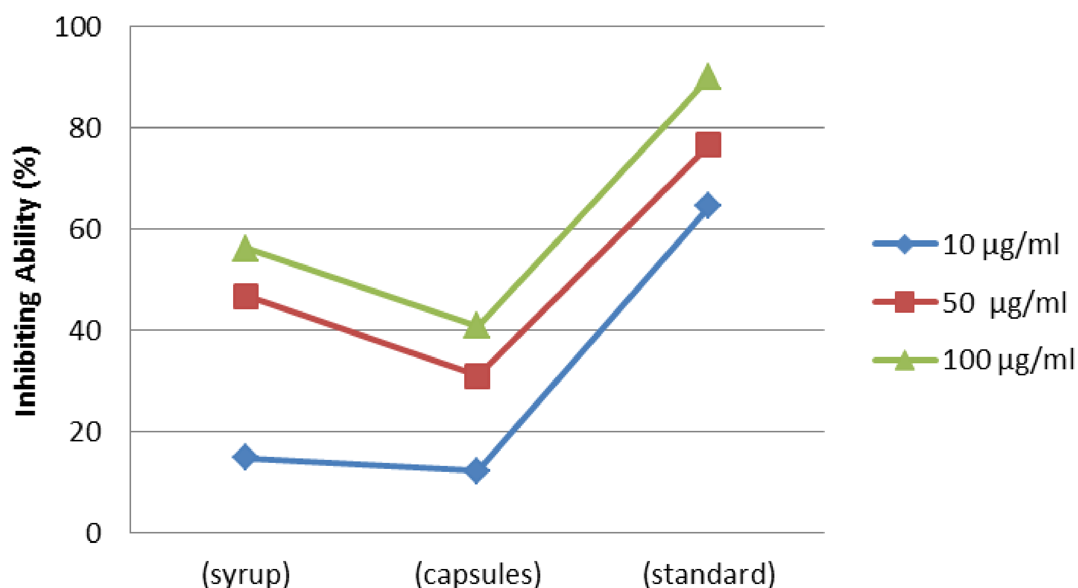

Figure 4. Lipoxygenase inhibiting activity by polyherbal formulation linkus.

ability was used. Absorption was noted on 517 nm due to odd electron. Good antioxidant activity was observed in both dosage forms. For determination of reducing activity Fe^{+3} to Fe^{+2} was the investigating point (Oyaizu, 1986). For antioxidant activity, the decreasing potential of a compound seems to be a substantial indicator (Meir et al., 1995). Percentage reducing ability was determined by the BHA standard formula and the results found good reducing ability in the poly herbal formulation.

Free radical and lipid per oxide play a vital role for the development of ulcer in human (Gutteridge, 1995). Linkus poly herbal formulation shows a good gastric protection. Herbal formulations have anti-inflammatory activity, including many disorders such as cough, chronic laryngitis and many others (Kapoor, 2000; Madras, 1993). Beside all the functions and activities linkus formulation shows good lipoxygenase inhibition activity (Figure 4).

Cough is the furthermost common today's symptom seen in overall family practice. Clinically, a cough is nearly a symptom of an underlying illness. It is significant to look beyond it to treat the cause and, hence achieve the maximal relief from cough and related symptoms. Studies have shown that there is an interaction between respiratory tract infections and antioxidant activities (Rubin et al., 2004; Gilliland, 2003) and oxidative stress present in blood due to respiratory infections (Gilliland et al., 2003). Lipoxygenase and leukotriene are the key factors for the inflammatory responses and respiratory distress (Wasserman et al., 1991). Multiple events suggested that lipoxygenase have strong relation in physiological event in respiratory tract infection (Holroyde, 1981; Weiss, 1982; Barne, 1984; Smith, 1985; Adelroth, 1986).

This study has shown the visible antioxidant activity, urease and lipoxygenase activity in poly herbal formulation Linkus cough syrup and lozenges (Figure 4). These dosage forms have contributing factors towards the indication cough as antioxidant and anti-inflammatory activity. These type of activity are due to free radical 2,2'-diphenyl-1-picryl hydrazyl, conversion of ferric into ferrous state, ammonia production using the indophenol method and hydroperoxides produced in the lipoxygenation reaction.

Conclusion

The poly herbal extract based lozenges and syrup were analyzed *in vitro* for anti-oxidant, urease and ACE inhibiting activity. Syrup was found to be more potent in comparison with lozenges but significant ability was found in contrast assessment with standard. It might be helpful for the reduction of respiratory tract infection and allied problems with minimum adverse/side effects.

Conflict of Interest

The authors have not declared any conflict of interest.

ACKNOWLEDGMENT

Thank you for my teachers and friends for moral support.

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Appendix 1. Linkus Syrup composition per 10 ml.

S.No	Ingredients	Quantity/10 ml
01	Adhatoda vasica – Bansa	600.00 mg
02	Piper longum – Filfil Daraz	100.00 mg
03	Cordia latifolia – Sapistan	100.00 mg
04	Glycyrrhiza glabra – Mulethi Extract	75.00 mg
06	Alpinia galangal – Khulanjan	50.00 mg
07	Viola odorata – Banafshan	25.00 mg
10	Onosma bracteatum – Gaozaban	100.00 mg
11	Methyl Paraben	10.928 mg
12	Propyl Paraben	2.168 mg
13	Sugar	7000.0 mg
14	Citric acid	20.00 mg
15	Glycerin	0.100 ml
16	Peppermint Oil	0.003748 ml
17	Clove Oil	0.001252 ml
18	Propylene Glycol	0.001668 ml

Appendix 2. Composition of Linkus Lozenges per lozenges.

S. NO.	Composition	Content (%)
1	Adhatoda vasica Nees.	1.2
2	Glycyrrhiza glabra L.	0.28
3	Piper longum L.	0.24
4	Hyssopus officinalis L.	0.12
5	Alpinia galanga (L.) Wild	0.12
6	Viola odorata L.	0.08
7	Mentha piperita L.	0.08
8	Sugar	q.s 100
9	Liquid glucose	40
10	Anhydrous citric acid	1.28
11	Talc	0.48
12	Mineral oil	0.176
13	Menthol	0.16
14	Eucalyptus Oil	0.12
15	Paraffin	0.04
16	Beeswax	0.04
17	Lanolin	0.04
18	White soft paraffin	0.024
19	Tablet essence for Orange lozenges	0.852
20	Tablet essence for Honey lemon lozenges	0.852

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