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Research Article

Toxicity, phytochemical content and antioxidant activity assessment studies for a standardized ethanolic fraction of palm oil leaf extract

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ABSTRACT: Palm oil is one of the main contributors to vegetable oil industry. Its fronds are always discarded as waste products. Nowadays, there are some studies focused the light on the biomedical importance of palm oil leaf extract as a comestible health product. In this study, ethanolic fraction of the extract was used. Its antioxidant activity was investigated by using reducing power assay, free radical scavenging activity, hydrogen peroxide inhibition assay and antilipid peroxidation assay. The polyphenols and flavnoids contents were determined. Using Folin Ciocaleau and Sakanaka tests. LD_{50} was determined using OCED protocol (Up and down procedure). The subchronic toxicity was assessed by feeding the extract in 3 different doses 0.5, 1 and 2 g/kg/day for 24 days. At the end gross and necroscopical examinations were performed. Results showed that POLE possesses an antioxidant activity and its safety limit is high. Overall, POLE can be used as a comestible health product to protect organs against injury.

KEYWORDS: Antioxidant, polyphenols, peroxidation, palm oil and free radicals.

INTRODUCTION

Ingestion of botanical products rich in polyphenols can reduce the mortality rate of cancer and cardiovascular diseases. Polyphenols possess radical scavenging properties and can be used as organs protective agents.[1]

Free radicals are generated due to the intracellular activities related to oxidative phosphorylation during energy production and phase I metabolic reactions during elimination of xenobiotics. They exert their deleterious effect through damaging the intracellular macromolecules as DNA, protein and lipids.[2]

The natural source of the botanical products and the desire of people to use them have pushed many researchers to investigate their prophylactic and therapeutic potential as health products. Moreover, there are lots of in vivo studies revealed that natural polyphenols are absolutely safe.[3] Although, some in vitro studies revealed that they are toxic in high doses. Nevertheless, the in vitro tests give false positive results due to the high concentration of the tested compound which is hardly attained in vivo.[4] Some recent studies revealed that polyphenols are Janus molecules, they do not merely act as antioxidants but they also act as inhibitors for some enzymes as cytochrome P450 and different intracellular kinases.[5]

Palm oil (Elais guinensis) is cultivated mainly in the tropical areas. It has a great deal of contribution in vegetable oil industry. The fronds are mostly discarded as waste products but nowadays the light was focused on their biomedical importance. Especially after discovering that they contain polyphenols, tocotrienols and carotenoids.[6]

The botanical products are classified into 3 categories according to their medical effect; supplementary food, medical and poisonous products.[7] POLE falls into the first categories of these products.[6] It is very important for nutritionists and herbalists to understand the full toxic and pharmacological effect of the product in order to avoid

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the possible toxic effect of their overconsumption.[7] The toxicological studies give a comprehensive apprehension about the effect of various products on human physiology and drug interaction.[8]

Assessment of the effect of the toxicant requires a high dose feeding of the test compound. The acute toxicity study aims to determine LD_{50} of the compound. LD_{50} is the dose in mg/kg (B.W) that causes 50% lethality. On the other hand, the chronic or sub-chronic toxicity study aims estimate the secondary stages of toxicity. It is concerned with organ damage and appearance of toxic signs and symptoms. These observations must be appended by biochemical and hematology laboratory tests in order to evaluate the possible secondary toxic effect of the product.[8]

Therefore, the study was concerned with the possible toxic effect of oral administration of ethanolic extract of POLE. In the toxicity study, $LD_{50,}$ no-observable effect level (NOEL) and no-observable adverse effect level (NOAEL) of the extract were determined.

METHODOLOGY

The extract

Palm oil leaves were collected and subjected to the freeze drying for 24 hours. Then the dried leaves were pulverized and the extract was prepared by soaking the powder with absolute alcohol 1:20 (w/v) for two days. Ethanol is the ideal solvent to extract the polyphenols as it has a good power to build intra-molecular bonds with them. The organic impurities were discarded by fractionation with chloroform. After that, the mixture was filtered and the residue was extracted twice. At the end, it was dried till the solvent was completely removed.

Preliminary phytochemical analysis

A preliminary qualitative analysis was performed to investigate the chemical nature of phytochemicals present in the extract. Phytotochemicals present in plants are alkaloids, phenolics (they include tannins, flavnoids, phenylpropanoids and other polyphenols), saponines or steroids.[9]

Qualitative detection of alkaloids

3 ml of the extract was mixed with 1 ml (1% HCL) solution. The mixture was heated for 20 minutes. Then it was cooled and filtered. At the end 2 drops of Mayer reagent was added to 1 ml of the filtrate Mayers reagent is a potassium mercuric iodide solution. It is a mixture of mercuric chloride (1.36 g) and of potassium iodide (5.00 g) in water (100.0 ml). Presence of a creamy precipitate is an indication of presence of alkaloids.[10]

Qualitative detection of phenols

Two drops of ferric chloride solution (5% w/v) was mixed with 1 ml of the extract. Appearance of a greenish precipitate indicates presence of phenols.[11]

Qualitative detection of flavnoids

Presence of the flavnoids was inferred from the yellow coloration of the mixture of 3 ml of the extract with 1 ml of 10% NaOH solution.[11]

Qualitative detection of tannins

Tannins were detected qualitatively after mixing of 1 ml of a freshly prepared 10% (w/v) ethanolic KOH solution with 1 ml of the extract. Appearance of white colored cloudiness is an indication of tannins presence.[11]

Qualitative detection of saponins (Frothing test)

Saponins have surface active properties that upon shaking the extract vigorously with water for 2 minutes and the mixture is warmed a froth resistant to warming appears which indicates presence of saponins.[12]

Tests for Coumarins

The crude plant extract (1g) was taken in test tube and covered with a filter paper moistened with dilute NaOH. The test tube was placed in boiling water for 5 minutes. The filter paper was then removed and was observed under UV light. Appearance of yellow fluorescence is an indication of presence of coumarins.[13]

Tests for Sterols and Terpenes

1 ml of the extract was mixed with 20 ml $CHCl_3$. After that, the $CHCl_3$ layer was dried over anhydrous Na_2SO_4. Then 5 ml of the $CHCl_3$ layer was mixed with 0.5 ml of acetic anhydride to which two drops of conc. H_2SO_4 was added. The gradual appearance of green, blue, pink colour was considered to be an indication for the presence of sterols, while development of pink to purple colours was an indication for the presence of triterpenes.[13]

Qualitative detection of steroids

Presence of steroids can be inferred by adding five drops of concentrated sulphuric acid to 1 ml of the extract. Red coloration of the mixtures indicates the presence of steroids.[13]

The preliminary phytochemical analysis of the extract showed presence of polyphenols including the flavnoids and tannins. Saponins and Terpenoids were detected while steroids, coumarins and alkaloids were absent. So accordingly the in vitro tests for antioxidant activity, total polyphenol content and hemolysis test for saponin were performed.

In vitro assessment of the contents and the antioxidant capacity of the extract.

Total phenol content

Total phenolic content was determined according to the modified method of (Singleton 1965). Polyphenols react with Folin Ciocalteu reagent in the presence of saturated sodium carbonate solution with maximum absorbance 725 nm (Analytikijena 200-2004 spectrophotometer). Standard curve was sketched using Gallic acid (0.01–0.4 mM). The results were expressed as mg of Gallic acid equivalents (GAEs) / g of extract.[14]

Determination of total flavonoid content

Flavonoids were determined according to the method described by Sakanaka *et al* (2005). 250 µl of the extract (1 mg/ml) was mixed with 1.25 ml of D.W and 750 µl of a 5% sodium nitrite solution. The mixture was left at room temperature for 6 min.. After that, 150 µl of 10% aluminum chloride solution was added and the mixture was again left for 5 min.. Then 0.5 ml of 1M sodium hydroxide was added and the volume was made up to 2.5 ml with D.W and mixed well. Finally, the absorbance at 510 nm was determined versus blank. A calibration curve was prepared using (+) catechin as a standard. Results were expressed as mg of (+) catechine equivalents (CE) per gram extracts.[15]

Free radical scavenging activity

The free radical scavenging activity of the ethanolic fraction of POLE was done by measuring the ability of the extract to quench 1, 1-diphenyl-2-picryl-hydrazil (DPPHº) free radical using method of *Shimada, fujikawa, Yahara and Nakamura* (1992). Eight times serial dilution were done for 1mg/ml solution of the extract then 3 cc of each was mixed with 1cc of 0.1M DPPHº dissolved in methanol. After half an hour, the absorbance was measured at 517 nm. The absorbance was plotted against the concentration. EC_{50} (Extract concentration of that inhibits 50% of the original DPPHº activity) was calculated. It was compared to that of vitamin C and butylated hydroxytoluene (BHT).[16]

Reducing power assay

Reducing power of the extract was measured according to method of *Oyaizu* (1986). In which different concentrations, prepared by serial dilution of 1 mg/ml solution of the extract, were mixed with the mixture of Prussian blue reaction (Malory reaction) according to the prescribed method. The reducing power of the extract is deduced from its ability to intensify the reaction and increase the absorbance at 700 nm.. A plot of extract concentration verses percent of absorption increase was sketched. IC_{50} which is the concentration of the antioxidant solution required to increase the intensity of absorption by 50% was calculated and compared with that of different concentrations of vitamin C and butylated hydroxytoluene (BHT).[17]

Hydrogen peroxide scavenging activity

It is determined by method of *Oktay et al* (2003). In which the ability of different concentrations of the extract prepared by serial dilution to quench hydrogen peroxide was measured using UV-spectrophotometer at a wavelength 230 nm. Percent of hydrogen peroxide scavenging ability was measured and a plot of this percent versus each concentration was sketched. EC_{50} was deduced from the plot for the extract and compared to that of vitamin C and butylated hydroxytoluene (BHT).[18]

Anti-lipid peroxidation assay

Anti-lipid peroxidation capacity was measured according to *Kimuya Y, Kubo M, Tani T, Arichi S, Okuda H*, in which different concentrations of the extract, prepared by serial dilution, were incubated with a rat liver homogenate mixed with ferric chloride which induces lipid peroxidation. Finally, the antilipid-peroxidation capacity of the extract was deduced by measuring the amount of MDA release with each concentration of the extract and compared with the control in which distill water was added instead of the extract.[19]

Hemolytic test for saponin content assessment

Hemolytic activity of the extract gives an indication about content of saponins. The method was adopted from (Mackie 1968) and (Kalinin 1996).[20]&[21] Citrated blood from rats was used in the experiment. Erythrocyte suspension was prepared by centrifuging the blood sample at 1000g for 15 min. They were washed and centrifuged three times in cold phosphate buffer saline (pH 7.4). Two ml of this packed cells suspension as diluted with the same buffer up to final volume 100 ml. Then 20 µl of 10 mg/ml of the extract was mixed with 1980 µl of erythrocyte suspension. Then the mixture was incubated for 1 hr. at room temperature and was centrifuge, Absorbance of the supernatant using visible light spectrophotometer at 540 nm was measured. different concentrations of standard Quillaja bark (S4876, Sigma-Alrich, st. Louis, MO) prepared by serial dilution method was used to make a standard curve. Results were expressed as mg equivalents of Quillaja bard/gm of pre extract.

Toxicity study

Animals

Female Sprague Dawley rats weighing 100±13 g obtained from the animals house facility/ Universiti Sains Malaysia. The animals were left for habituation in the transit room facility of the school of pharmacy for one week prior to starting the experiment. The animals were kept at 25 ± 2 C under 12 hours light / dark cycle. They were fed with the standard rodents chow with free access to water and standard rodent's pellets. The lyophilized extract was suspended in water and vortexed prior to oral administration. The animals were maintained and handled according to the recommendations of USM ethical committee.

Subacute oral toxicity study

In this study, fixed dose procedure (FDP) according to the OECD guideline was followed.[22] 5 groups of female SD rats (n=10) were used in the study. The first groups served as controls (given water alone as a vehicle) while the rest were fed with 0.5, 1, 3 and 5 g/kg (B.W) of ethanol extract of POLE for 24 days respectively. The rats were observed for morbidity and appearance of toxic signs and symptoms throughout the study period. Food and water uptake were monitored as well. At the end, the rats were fasted overnight (12 hours before blood collection). Then blood was taken via cardiac puncture for biochemistry and hematology study. Half of the survival rats (n=5) were sacrificed to obtain the relative weight of the organs as liver, spleen, kidney, heart, lung and spleen and to examine the organ abnormalities.

Recovery period

After that, half the survival rats are returned back to the cages and monitored for another 24 days for the late toxicity. All of them had free access to water and food. Their uptake was recorded as well during the recovery period. At the end the rats were also sacrificed and were subjected to microscopy and biochemical study to observe the possible organ damage and change in their relative weight.[22]

Acute oral toxicity

This study is used to estimate LD_{50}. It is estimated using OCED guideline for testing acute toxicity of chemicals (Acute oral toxicity- up and dawn procedure (UPD)).[22] One of the two UPD based protocols (main and limit test protocols) could be adopted. The limit test protocol is conducted when the experimental information indicates that the test material is relatively non toxic, hence it was used in the study. 5 g/kg was chosen as the upper bound limit. The animals were fed sub sequentially (one animal each day) with 5 g/kg of the extract. They were monitored for morbidity and appearance of adverse effect within the first day. If the reversals have had occurred, the main test protocol would have been adopted otherwise the LD_{50} should have been reported as being higher than 5 g/Kg.

RESULTS

Preliminary phytochemical analysis

The preliminary phytochemical analysis of the extract showed presence of polyphenols including the flavonoids and tannins. Saponines were detected also. Steroids and alkaloids were absent in the extract.

Total polyphenols and flavonoids content

The analyses showed that the concentration of polyphenols was 52.4 mg GAE (Gallic acid equivalents)/g of the pure powdered extract while flavonoids concentration was 15.4 mg catechine equivalents/g of the pure powdered extract.

Antioxidant activity of the extract

Figures 1, 2 and 3 depict the free radical scavenging, hydrogen peroxide scavenging and reducing power activities of the extract as compared to both vitamin C and butelated hydroxytoluene (BHT). All of these tests showed weaker antioxidant activity for the extract as compared to the mentioned standard antioxidants. POLE intensified the color of Prussian blue reaction with IC_{50} = 41 µgm/ml as compared to BHT and vitamin C whose IC_{50} = 2 &

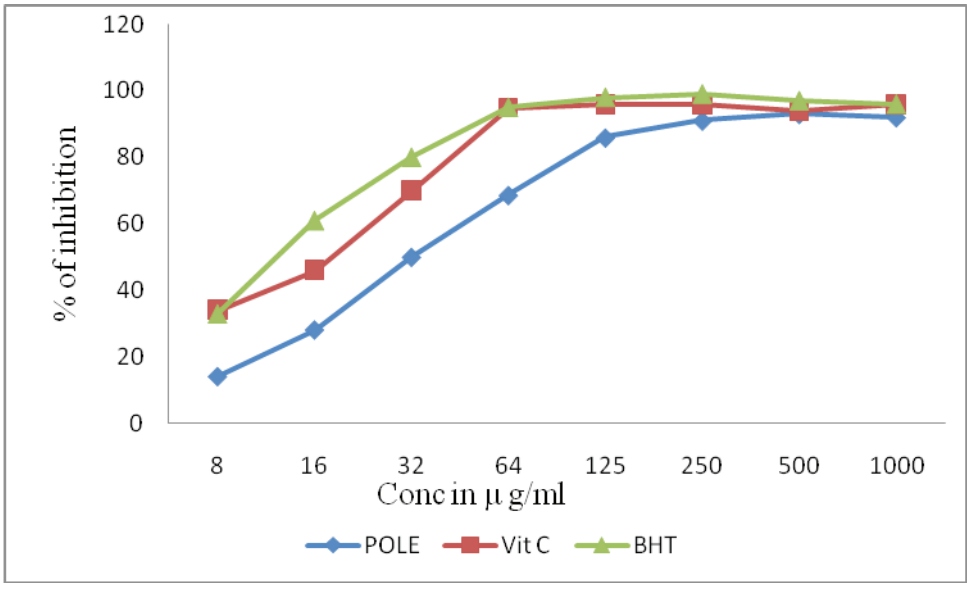

Figure 1: In vitro evaluation of free radical scavenging activity of the extract as compared to that of vitamin C and BHT. The concentrations were ranging between 8 -125 µ g/ml. The data fit the linear equation: Y=0.57X+21 (R^2=0.87) for POLE and were fitting the equations Y=1.075X+29 (R^2=0.97) and Y=0.98+37 (R^2=0.83) for both vitamin C and BHT respectively.

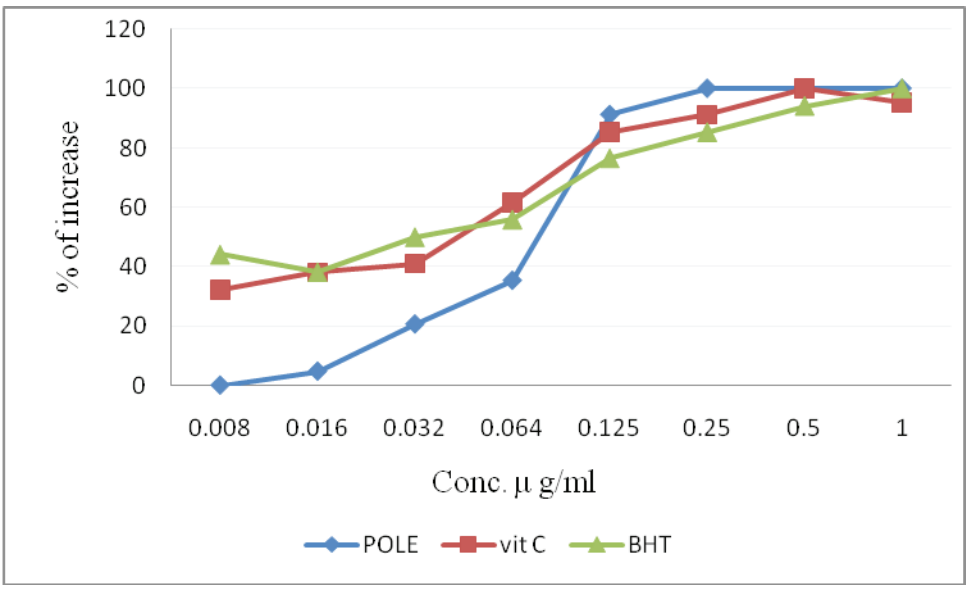

Figure 2: In vitro evaluation of reducing power for the extract as compared to that of vitamin C and BHT. The concentrations were ranging between 8-1000 µ g/ml.

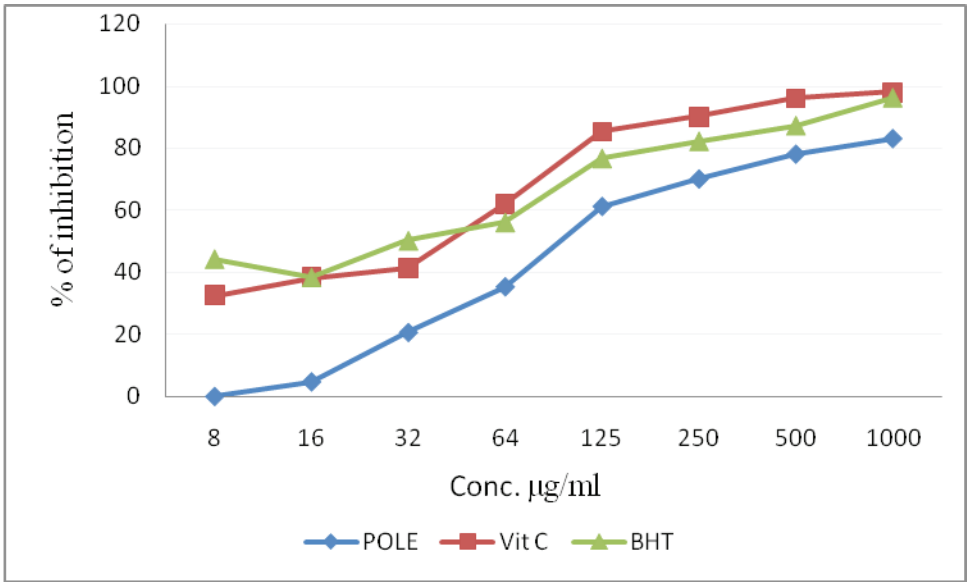

Figure 3: In vitro evaluation of hydrogen peroxide scavenging activity of the extract as compared to that of vitamin C and BHT. The concentrations were ranging between 8 -125 µ g/ml. The data fit the linear equation: Y = 0.76X + 7.25 (R^2 = 0.98) for POLE and were fitting the equations Y = 0.45X + 29.5 (R^2 = 0.98) and Y = 0.3 + 38.3 (R^2 = .95) for both vitamin C and BHT respectively.

4 µgm/ml respectively. It showed a concentration dependent antiradical activity by scavenging DPPH with a EC_{50} = 35 µgm/ml as compared to BHT and vitamin C whose EC_{50} = 11 & 18 µgm/ml respectively. Moreover, the extract showed a Hydrogen peroxide scavenging activity with EC_{50} = 72 µgm/ml as comparedvto vitamin C and BHT whose EC_{50} were 40 and 32 µgm/ml respectively.

Figure 4 shows the results of the anti-lipid peroxidation activity. There was a potential of POLE to interfere with Fe^{3+} induced lipid peroxidation as it was evidenced through suppressing the release of MDA after incubating the homogenate with the extract. EC_{50} of the extract was 140 µg /ml as compared to vitamin C whose EC_{50} was 28 µg/ml.

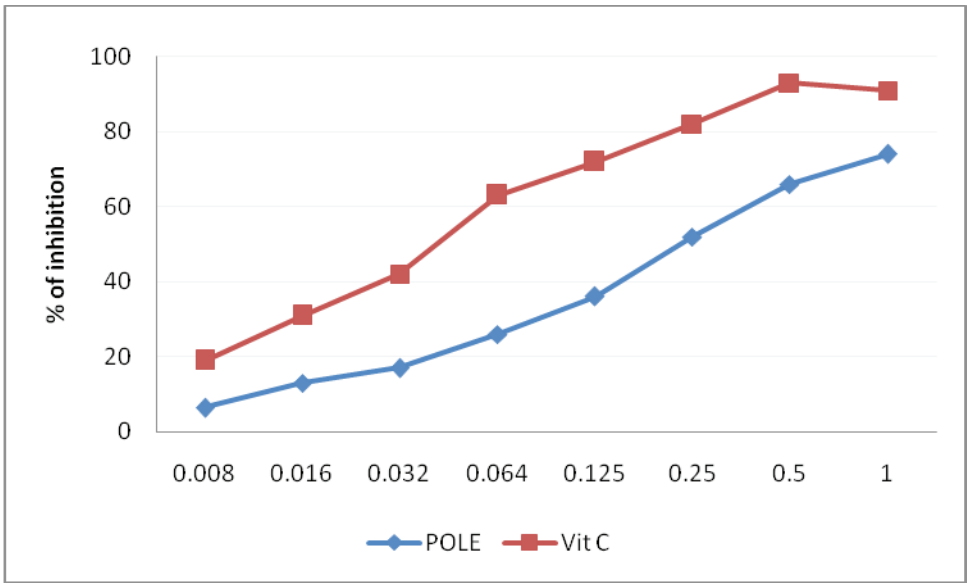

Figure 4: In vitro evaluation of antilipid peroxidation inhibition activity of the extract as compared to that of vitamin C. The concentrations were ranging between 8 µg/ml – 1 mg/ml. The data were fitting the linear equations: $Y = 65X + 20.3$ ($R^2 = 0.79$) and $Y = 59.9X + 46.6$ ($R^2 = 0.6$) for POLE and vitamin C respectively.

Hemolytic test for saponines

The results showed that saponines concentration was 0.8 mg equivalent of Quillaja bark / g of pure extract.

Subacute toxicity (28 days treatment)

Oral administration of POLE for 28 days did not produce any change in the behavior, respiration and neuronal response in the tested rats. Food and water consumption and body weight were not altered in all the groups as compared to both the positive and negative controls (table 3). Feeding of 0.5,1 and 2 g/kg/day orally of POLE for 28 days did not produce any effect on liver functions, renal functions, metabolic and hematologic status as it is obvious from results of the biochemistry study (table 1 and 2). Results of the relative weight of the organs showed a slight increase in the weight of liver for the positive control group and for the groups fed with different doses of the extract. On the other hand, the relative weight of the other organs was not affected (table1).

The 24 days recovery period

During the recovery period, there was no any group showed delayed adverse effect or lethality observed. Food and water consumption were not affected as well (table 4). The gross necroscopical findings at the end of the recovery period did not show any change in the external view of the organs and in the relative organs weight in all the treated groups.

Acute oral toxicity

The acute oral study showed that the extract is absolutely safe with $LD_{50} < 5$ g/Kg.

DISCUSSION

In this research, the chemical nature, antioxidant activity and the safety limit of the extract were investigated thoroughly. The antioxidant activity was assessed by measuring the potential of the extract to scavenge the free radicals, reduce them and interfere with the cascade of lipid peroxidatrion reaction. In the free radical scavenging activity assay, DPPH (1, 1-diphenyl-2-picryl-hydrazil) was used. DPPH is a free radical molecule with violet pink color. Its color fades when it is subjected to antioxidants. The test is highly reproducible and gives a comparable profile of the scavenging activity to that of other free radical scavenging activity tests as ABTS test.[16] &[23]

In the reducing power assay, potassium ferrocyanide reacts with ferric iron to form ferric ferrocyanide which gives the Perlis Prussian blue color. Ferric ion of ferric ferrocyanide is reduced in the presence of any reductant to ferrous ion. This intensifies the color of the Perlis Prussian blue complex. The test gives an idea about the potential of the extract to donate electrons to free radicals.[23]&[24]

In the hydrogen peroxide scavenging activity, effect of incubating hydrogen peroxide with the antioxidants was traced through tracing the absorbance of hydrogen peroxide at 230 nm. Hydrogen peroxide is one of the reactive oxygen species released during the cascade sequential reaction of free radical generation. Reactive oxygen species (ROS) are released due to the cellular metabolic activity. The body has a surveillance mechanism traces mops these species outside the body. ROS are released when this balance

Table 1: Results of biochemistry tests on the blood samples of all the treated groups at the end of the treatment period for the subacute toxicity study. Results did not show any statistical significance among the treated groups.

Biochemical parameter	Negative control	Positive control	P0.5	P1	P3	P5
ALT (I.U/L)	32±4.2	38±5.1	30±4.6	31±3.9	28±4.6	29.5±5.1
AST (I.U/L)	77±5.1	89±6.1	71±5.3	68±4.8	69±5.1	65±6.8
ALP (I.U/L)	281±58	320±68	271±48	265±52	268±53	262±48
GGT I.U/L)	0.7±0.35	0.68±0.31	0.65±0.3	0.62±0.41	0.64±0.33	0.58±0.31
T. Bilirubine (µmol/L)	1.71±0.15	1.92±0.18	1.65±0.2	1.68±0.15	1.72±0.16	1.69±0.18
CHO (mmol/L)	1.1±0.2	0.95±0.09	0.9±0.85	1.0±0.1	0.85±0.11	0.92±0.09
T.G (mmol/L)	0.66±0.1	0.69±0.12	0.61±0.09	0.58±0.11	0.60±0.09	0.57±0.10
Total protein (g/L)	70±5.8	66±6.2	62±5.8	64±6.3	66±5.9	62±4.8
Albumin (g/L)	34±5.3	29±5.9	29±5.3	30±5.8	32±6.1	31±4.2
Ser. Creatinine (µmol/L)	67.2±8.8	65±7.8	70±6.9	62.2±7.8	65±6.9	61±6.1
Ser. Urea (mmol/L)	17±3.5	23±3.8	20±4.1	19±3.9	20±4.5	22±6.4
Uric acid (µmol/L)	12.3±1.34	10.9±1.42	11.2±1.32	10.9±1.12	11.5±1.3	10.8±1.4
Ser Na^+(mmol/L)	141±9.1	144±8.5	140±9.5	138±8.5	136±7.8	139±8.2
Ser K^+(mmol/L)	3.8±0.45	3.6±0.42	3.5±0.38	4.1±0.36	3.8±0.42	4.1±0.41
Ser Ca^+(mmol/L)	2.1±0.1	2.2±0.15	2.1±0.12	2.3±0.18	2.4±0.21	2.5±2.3
Ser Mg^+(mmol/L)	0.88±0.08	0.91±0.07	0.82±0.08	0.90±0.07	0.085±0.08	0.91±0.09
Cr Cl. ml/min/ 100 g (B.W)	0.18±0.02	0.16±0.03	0.19±0.02	0.14±0.01	0.11±0.008	0.16±0.03
Abs exc. Na^+ (ml/min/100 g (B.W)	0.016±0.00	0.014±0.00	0.021±0.00	0.016±0.00	0.016±0.00	0.018±0.002
Abs exc. K^+(ml/min/100 g (B.W)	0.05±0.005	0.06±0.004	0.08±0.007	0.06±0.006	0.06±0.003	0.08±0.007
FE Na^+	0.53±0.06	0.52±0.05	0.56±0.05	0.57±0.08	0.50±0.07	0.54±0.07
EF K^+	50±6	60±8	56±7	53±56	60±7	59±7
Chloride (mmol/L)	98.3±3.9	100±4.1	95±3.9	97.2±4.1	101±5.1	99.2±4.2
Abs. exc. Cl^-	0.01±0.01	0.01±0.01	0.01±0.01	0.01±0.01	0.01±0.01	0.01±0.01
FBG (mmol/L)	5.7±1.1	5.6±0.85	6.1±1.1	5.8±1.2	5.7±0.9	5.5±0.85
Inorganic phosphorus (mmol/L)	2.4±0.2	2.2±0.15	2.5±0.21	2.6±0.34	2.5±0.23	2.4±0.21
Sp. Gr. Urine	1.03±0.01	1.02±0.01	1.01±0.01	1.03±0.01	1.03±0.015	10.2±0.01
UFR (µL/min/100 g (B.W)	2.62±0.28	2.44±0.1	3.04±0.12	2.51±0.14	2.43±0.15	2.22±0.22

Table 2: Results of hematology tests on the blood samples of all the treated groups at the end of the treatment period for the subacute toxicity study. Results did not show any statistical significance among the treated groups.

Parameter	Negative control	Positive control	P0.5	P1	P3	P5
HB (g/dl)	15.1±0.8	16.2±0.65	14.3±0.9	15.2±0.75	15.6±0.82	15.9±0.82
RBCs count (10^{12} cell/L)	7.5±0.4	7.8±0.5	8.2±0.62	6.9±0.52	7.3±0.65	7.4±0.71
Hct	40.3±2.5	35.2±3.1	37.5±3.6	38.2±3.4	39.1±4.1	41.2±3.2
MCH (pg)	20.1±0.5	19.2±0.45	18.2±0.38	18.2±0.42	18.4±0.45	19.2±0.47
MCHC (g/dl)	34.5±1.5	33.6±1.7	32.5±1.8	35.6±2.1	34.1±1.6	33.5±1.9
MCV (fL)	55.5±1.9	54.6±1.8	60.1±2.1	57.2±2.5	56.2±1.8	58.3±1.9
WBCs count (10^9 cell/L)	8.5±2.3	8.0±2.1	7.8±2.8	7.5±2.6	6.8±3.1	7.6±2.6
Neutrophiles %	18.6±4.5	21.2±4.2	22.5±5.1	19.2±4.3	20.5±4.1	19.2±3.8
Lymphocytes %	78.5±4.8	73.5±4.8	74.5±4.5	76.2±4.5	73.2±3.5	76.5±3.8
Monocytes %	2.5±0.85	3.1±0.9	2.8±0.85	2.6±0.72	5.2±1.1	2.8±0.82
Eosinophiles %	1.7±0.6	1.5±0.84	1.3±0.72	1.6±0.85	2.1±0.75	1.8±0.68
Basophiles %	0.0±0.0	0.5±0.08	0.2±0.05	0.4±0.07	0.35±0.06	0.40±0.05
Platelets count (10^9 cell/L)	900±98.5	980±92.5	890±88.3	657±72.3	723±69.2	823±72.1
MPCV	4.2±0.28	3.8±0.32	3.6±0.35	4.4±0.36	4.2±0.29	0.37±0.38
Prothrombine time	13.1±0.9	11.8±1.1	12.5±1.3	14.1±1.6	13.5±1.8	10.9±0.54

Table 3: Results of relative organ weights of all the treated groups at the end of the treatment period for the subacute toxicity study. Results did not show any statistical significance among the treated groups.

Relative organ weight	Negative control	Positive control	P0.5	P1	P3	P5
Liver	2.73±0.07	2.70±0.06	2.68±0.11	2.85±0.09	2.72±0.1	2.88±0.1
Kidney	0.41±0.02	0.40±0.02	0.38±0.03	0.37±0.04	0.40±0.02	0.39±0.03
Heart	0.31±0.015	0.32±0.018	0.33±0.017	0.30±0.02	0.32±0.02	0.31±0.015
Lungs	0.53±0.02	0.51±0.015	0.49±0.025	0.48±0.02	0.46±0.02	0.51±0.03
Spleen	0.34±0.017	0.31±0.013	0.33±0.015	0.31±0.02	0.32±0.013	0.30±0.011

Table 4: Body weight (g), water uptake (ml/day) and food uptake (Kcal/day/100 g) through out the treatment period for the subacute toxicity study. Results did not show any statistical significance among the treated groups.

Group	Day 0	Day 8	Day 16	Day 24
		Body weight (gm)		
Negative control	102±1.85	107.8±2.9	118.3±6.5	131.2±6.5
Positive control	101.3±1.75	106.3±2.3	115.4±5.8	131.0±5.9
P0.5	100.5±2.1	106.1±2.8	121.2±5.8	134.3±7.2
P1	101±3.1	107.2±4.2	120.3±6.2	129.3±6.5
P3	102±4.5	109.9±3.9	121.2±6.7	126.2±5.8
P5	100±3.8	105.2±3.3	114.2±4.9	121.8±5.6
		Water consumption (ml/day)		
Negative control	22.5±1.1	25.5±1.2	24.5±1.3	24.5±1.4
Positive control	20.5±1.2	22.5±1.6	23.5±1.5	25.6±2.1
P0.5	24.3±2.0	24.2±1.4	24.5±1.4	25.8±1.4
P1	25.5±1.75	26.3±1.5	26.5±1.9	23.5±1.6
P3	23.5±1.2	28.5±1.6	25.5±1.2	26.3±1.4
P5	22.5±1.15	24.3±1.5	24.5±1.4	25.5±1.5
		Food uptake (Kcal/day/100 g)		
Negative control	28.4±2.1	27.0±3.0	24.6±2.2	268±3.2
Positive control	21.4±1.3	23.7±2.2	21.4±1.3	22.1±2.2
P0.5	27.5±2.4	27.1±1.85	22.4±1.95	24±2.3
P1	25±3.6	23±2.8	26.6±2.5	25.2±2.5
P3	26±2.7	30±2.4	27.2±3.1	24.5±2.8
P5	23±3.9	23±1.9	25±2.5	26.1±2.3

Table 5: Body weight (g), water uptake (ml/day) and food uptake (Kcal/day/100 g) through out the 24 days recovery period after the subacute toxicity induction. Results did not show any statistical significance among the treated groups.

Group	Day12	Day 24	Day12	Day 24
	Body weight (gm)		**Water consumption (ml/day)**	
Control	142±1.85	152.8±2.9	19.5±1.1	21.5±1.2
P0.5	143.5±2.1	150.1±2.8	21.3±0.9	20.2±1.4
P1	139.±3.1	148.2±4.2	22.5±0.85	21.3±0.9
P3	142±4.5	155.9±3.9	24.5±1.2	23.5±1.1
P5	138±3.8	151.2±3.3	19.5±1.15	20.3±1.2
	Food uptake (Kcal/day/100 g)		**Liver**	**Heart**
Control	30.7±2.6	31.2±2.6	2.73± 0.07	0.31±0.015
P0.5	29.4±1.8	30.7±1.2	2.72±0.10	0.32±0.018
P1	30.3±2.8	27.3±3.0	2.68±0.11	0.33±0.017
P3	26.1±1.2	29.6±2.4	2.85±0.09	0.30±0.020
P5	25.3±2.1	24.3±2.3	2.88±0.10	0.32±0.021
Group	**Kidney**	**Lung**	**Spleen**	**Lethality**
Control	0.41±0.02	0.53±0.02	0.34±0.017	0/5
P0.5	0.38±0.03	0.51±0.01	0.31±0.013	0/5
P1	0.37±0.04	0.49±0.03	0.33±0.015	0/5
P3	0.40±0.02	0.48±0.02	0.32±0.013	0/5
P5	0.39±0.03	0.51±0.03	0.30±0.011	0/5

mechanism is disturbed. Ingestion of more antioxidants rich products is required to scavenge the redundant hydrogen peroxide.[19] Free radicals have a powerful predilection to snatch electrons from cell membrane lipids. These lipids are endowed with a great deal of unsaturated free fatty acids. This changes fatty acid into fatty acid radicals. Fatty acid radicals are highly unstable and tend to react with another oxygen molecule to form lipid peroxyl radicals. The last can further attack other free fatty acid molecule to induce a chain propagation reaction of free radicals generation and lipid peroxidation. If this process proceeds progressively, membranous structures would be damaged leading to cellular degeneration and necrosis. Ferric ion is one of the inducers of lipid peroxidation. It has a tendency

to be reduced to ferrous ion inducing a famous reaction known as Fenton reaction. In antilipid peroxidation assay, lipid peroxidation was triggered through incubating ferric ion with liver homogenate which is endowed with a great deal of membranous unsaturated free fatty acids. Malonyldialdehyde release was used as a lipid peroxidation marker. The antioxidant power of the extract halted progression of lipid peroxidation and suppressed malonyldialdehyde release. This potency can be an indicator for the polyphenol content and the potency of these polyphenols to antagonize Fenton reaction and the progression of lipid peroxidation.[19]&[25]

LD_{50} describes only one aspect of toxicity which is the lethality after feeding a high dose of the extract. It is the toxic dose of the extract required to kill half the tested animals. The precise LD50 could not have been determined in the research as it was higher than 5 g/kg. Till this dose limit, no adverse effect was seen as well. According to (Ecobichon 1995), any test compound fails to produce adverse effects at a dose limit exceed 5 g/kg is considered practically non toxic.[26] Moreover, the chronic toxicity study did not show any observable change on the animals after feeding the animals till the 5 g/kg/day dose of POLE for 28 days. Thus this limit can be the no observable adverse effect level (NOAEL). On the other hand, the chronic toxicity study did not show any significant change in both the biochemical and hematological parameters. So feeding with 5 g/kg/ day feeding of the extract for 28 days is considered to be within the (no observable effect level-(NOEL)). The increase in the relative weight of liver without showing any abnormalities in the liver function test does not indicate a possible hepatotoxicity. Liver congestion and overgrowth of hepatocytes were reported after feeding the animals with high doses of herbs or drugs. Some previous studies revealed that loading the liver with nutrients and chemicals triggers the hepatocytes to synthesis more macromolecules proteins and triggers the smooth endoplasmic reticulum to proliferate. This increases the relative organ weight of liver. According to (Schulte-Hermann 1979), the increment in the relative weight of liver in short term experiments is not related to a pathogenic change but it is related to cellular hyperplasia and hyperatrophy.[27] This finding was manifested in our experiment as the relative weight of liver was normal in the rats passed the recovery period after stopping the extract feeding.

CONCLUSION

Overall, our study revealed that the extract possesses an antioxidant action which may is attributed to its polyphenol content. It does not have any toxic potential that compromise its use as a medicinal health product.

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