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Effect of Ellagic Acid Extracted from Walnut (*Plukenetia Conophora)* used in the Treatment of Antioxidant Imbalance in Female Infertility

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Abstract
Objective: *This study was carried out to investigate the effects of Plukenetia conophora on female infertility that involves the qualitative phytochemical analysis, in-vitro and in-vivo antioxidant property of the aqueous ethanolic extract and ellagic acid extract of Plukenetia conophora leaves. This research was promptedbased on the traditional claims that, it is used for the treatment of infertility in females.*
Methods: *Qualitative phytochemical screening of the aqueous ethanolic extract was performed. In-vitro antioxidant assay of Total Antioxidant Capacity (TAC) was determined. In-vivo antioxidant activity investigated was catalase (CAT). Aqueous ethanolic extract and Ellagic acid extract of Plukenetia conophora were administered orally for 30 days to the female albino rats in the groups, including female rats induced with temporary infertility drug(POSTINOR) for 4 days before the administration of the extracts. Testosterone was determined using competitive microplate immunoassay.*
Results: *The phytochemical analysis of the extracts showed the presence of steroids, phlobatannins, saponins, tannins, flavonoids, resins. The aqueous ethanolic extract showed inhibition of 12.72% and ellagic acid extract inhibition was 77.95% TAC at 100µg/ml concentrations. Also, the aqueous ethanolic extract caused reduction of the activities of CAT (0.317U/ml) and ellagic acid extract caused reduction of the activities of CAT (0.591U/ml) compared with control group. The results clearly demonstrated that Plukenetia conophora possessed good antioxidant activity.*
Significant increase was observed in testosterone level of both aqueous ethanolic extract group and ellagic acid extract group as compared with control group. Also, there was significant increase in triglyceride level of both the induced infertile group and ellagic acid group as compared with the control group. There was a significant increase in cholesterol level of the induced infertile group as compared with the control group.
Conclusion: *It was concluded that Plukenetia conophora leaves may be used as female fertility agent.*
Keywords: *Infertility, Plukenetia conophora, Reactive Oxygen Species.*

1. Introduction

Infertility is defined as the inability to conceive after trying for at least one year without success. There are so many confounding factors that can cause infertility. The major causes of female infertility are due to ovarian dysfunction, polycystic ovarian syndrome.

Significant scientific evidence has shown that, under situations of oxidative stress reactive oxygen species are generated and the balance between antioxidant (reduction) and oxidation is believed to be a critical concept for maintaining a healthy biological system (Davies, 2000).

Reactive Oxygen Species (ROS) are produced during normal cellular function. ROS include hydroxyl radicals (OH), Superoxide anion, hydrogen peroxide (H_2O_2). They are transient species due to their high chemical reactivity that leads to oxidation of some enzymes (Mátes *et al.*, 1999). ROS can affect a variety of physiological function in the reproductive tract and excessive levels can result in pathologies affecting female reproduction.

Under normal conditions, antioxidants act to oppose ROS production, scavenge existing free radicals and promote the repair of ROS induced damage to cell structures. Non-enzymatic antioxidants include vitamin C and vitamin E. Enzymatic antioxidant includes catalase. The degree of antioxidants defense present is often expressed as total antioxidant capacity (TAC). A disruption in the delicate between antioxidants and pro-oxidant molecules can result in oxidative stress (OS). OS arises when the generation of Reactive Oxygen Species and other radical species overrides the scavenging capacity by antioxidants either due to the excessive production of ROS or an inadequate availability of antioxidants.

African Walnut (*Plukenetia conophora)* is a member of Euphorbiaceae family. It is a climber found in wet parts of Eastern, Western Nigeria and other part of West Africa. Its habitat is usually large trees; the fruit are greenish with four round seeds in each fruit. The seed testa is hard, and the cotyledons are white in colour. The fruits are edible, the plant is medicinal and used for various purposes (Burkill, 1984). The leaves, barks and fruits of *P. conophora* are used medicinally purpose which include giddiness, thrush, toothache, and as an antidote to snake bite (Odugbemi, 2008).

In the southern Nigeria ethnomedicine, African walnut is used as female fertility agent and in the treatment of dysentery (Ajaiyeoba and Fadare, 2006). The leaves extract of *Plukenetia conophora* possess antibacterial (Ajaiyeoba, 2012) and antifungal activities (Ajaiyeoba and Fadare, 2006). Walnut extracts possess antioxidant property (Herbet *et al.*, 1998) and reduce Reactive Oxygen Species caused by Oxidative Stress.

Polyphenolic compound such as flavonoids are widely distributed in plants which have been reported to exert multiple biological effect including anti-inflammatory activities (Irshad and Chaudhan, 2002; Haug *et al*., 2005).

However, traditional claims have proven that ethanolic extract of the plant leaves can be used as remedy for infertility in females. While in female, the plant extract is taken to reduce oxidative stress which could affect fertilization processes and even improper implantation of the embryo in the womb leading to abortion (Jauniaux *et al.,* 2004).The purpose of this study was to investigate the effect of ellagic acid extract and aqueous ethanolic extract obtained from *Plukenetia conophora* used as therapy of antioxidant imbalance in infertile females using rat model, the active component of ellagic acid extract and aqueous ethanolic extract of *Plukenetia conophora* include tannins, phenols, saponins, flavonoids.

2. Research Methods

2.1.1Collection of plant materials: The leaves of *Plukenetia conophora* were collected from Owo town in Ondo state. The plant part was identified at the University of Lagos Herbarium by O.O Oyebanji where a voucher specimen (Ref No: LUH 7026) was deposited for future reference.

2.1.2 Preparation of plant: The plant leaves were cleaned and air-dried in room temperature for 3weeks. The dried *P. conophora* leaves were powdered and weight of powdered leaves was 300g. The powdered leaves were stored in air-tight container.

2.1.3 Aqueous ethanolic extract: A total of 200g powdered leaves were soaked with 700ml of ethanol and 300ml of water 70:30 for 72 hours. The extract was filtered using white muslin cloth and filtrate was dried using water bath (model DK420). Weight of aqueous ethanolic extract - 36.2g. The dried extract was stored in an air-tight

container and kept in refrigerator till time of use (Ayola et al., 2011).

2.1.4 Ellagic acid extract: Sixty grams of dried plant sample was transferred to tubes containing 49.5 mL of methanol and 5 mL of 6 M H_{CL} (Lei, 2002). The tubes were closed and the content was incubated for 72 h at 90°C. After cooling, this material was filtered through a Whatman No.1 filter paper in a vacuum system. The yield obtained was kept in air tight container and kept in refrigerator till time of use.

2.1.5 Phytochemical screening of *Plukenetia conophora*and the Ellagic acid extract

The ellagic acid extract and the ethanolic extract of *P.conophora* was subjected to both qualitative and quantitative phytochemical screening using standard procedures to identify the constituents as described by Sofowara (1993), Trease and Evans (1989), Harborne (1973) Van-burden and Robinson (1981) Bohm and Kocipai-abyazan (1994), Obadoni and Ochuko (2001).

2.3.8 Toxicity test: Six rats were selected for the toxicity test (two per group) and starved overnight. Extract doses of 10, 100 and 500mg/kg body weight were administered to each animal groups respectively. The animals were observed for toxicity signs and mortality for 72 hours. At 500mg/kg body weight, animals became weak and mortality was observed in this group.

2.3.9 Experimental protocol

A total of 42 female albino rats (100-120g) used for the study were obtained from University of Ilorin and acclimatized for 14 days. The rats were divided into six groups of six rats each. Postinor was used to induce infertile state in the rats. Oral administration was done using oral cannula and all rats were fed standard diet and had water *ad libitum*, while the treatments for each group were as follows:

2.4.0 Control groups

Group 1 (Control): Rats were given feed and distilled water only, throughout the experimental period.

Group 2 (Postinor only): Rats were administered 1*ml* of postinor at a dose of 0.0145mg/kg body weight orally for 4 days only.

Group 3 (Aqueous ethanolic extract-only): Rats were administered with 1*ml* of extract only for 30 days at a dose of 100mg/kg.bw.

Group 4 (Ellagic acid extract): Rats received ellagic acid extract at a dose of 1*ml* for 14days orally.

Group 5 (Pregnant group): To test for the effectiveness of the extracts in the treatment of infertility, three rats weighing 120g each were separated from the control groups (distilled water, aqueous ethanolic extract and ellagic acid). The estrus cycle of the rats was determined by collecting mucous like fluid from the vaginal of the rats using swap containing normal saline and then viewed under a monocular microscope to determine the morphology of the cells before mating with two normal male rats weighing 200g in a separate cage and observed for conception throughout the period of experiment.

2.4.1 Test groups

Group 6 (Postinor + extract): Rats received 1ml of postinor at a dose of 0.0145mg/kg body weight orally for 4days orally and were thereafter administered with plant extract at a dose of 100mg/kg.bw(1*ml*) for 26 days orally.

Group 7 (Postinor + ellagic acid extract):Rats received 1ml of postinor at a dose of 0.0145mg/kg body weight orally for 4 days orally and were thereafter administered ellagic acid extract at a dose of 1 *ml* for 26 days orally.

2.4.2Animal sacrifice and sample collection:24 hours after the last administration, the animals were sacrificed by cardiac puncture and blood samples collected in plain well labeled bottles. These were later centrifuged for 15mins at 1000rpm. The supernatant obtained was stored frozen until used for biochemical and hormonal analysis.

2.4.3 Procedure for testosterone determination

Testosterone level was determined using competitive microplate enzyme immunoassay. The essential regents required for an enzyme

immunoassay include antibody, enzyme-antigen conjugate and native antigen. Upon mixing biotinylated antibody enzyme-antigen conjugate and serum containing the native antigen, a competition reaction conjugate for a limited number of antibody binding sites interaction.

The simultaneous reaction between the biotin attached to the antibody and the streptavidin immobilized on the micro-well occurs. This effects the separation of the antibody bound fraction after decantation. Sufficient wells for calibrators, control and the test samples were properly placed in duplicate.50µl of each calibrator, control and test samples was added to the corresponding labeled wells in duplicate followed by addition of 100µl of conjugate working solution. The solution was properly mixed and incubated for 1h at room temperature. Each well was washed properly with 245µl of diluted washed buffer and the plate firmly tapped against absorbent paper to ensure it was dry. 150µl of tetramethylbenzidine (TMB) substrate was added to each well and incubated for 15 minutes at room temperature before the addition of 50µl stop solution. The absorbance was read at 450nm within 20 minutes after addition of the stop solution.

$$\text{Testosterone concentration} = \frac{\text{Standard}}{\text{Test}} \times \text{Concentration (ng/ml)}$$

2.4.4Assay of ROS Scavengers level (in-vitro) using various methods:

Total Antioxidant Capacity (TAC): The total antioxidant capacity of the extract was determined using phosphomolybdate method as described by Prieto, et al., (1999). The total antioxidant capacity of the extract was determined with phosphomolybdenum using α- tocopherol as the standard. An aliquot of 0.1*ml* of plant extract (100µg) solution was combined with 1.0*ml* of reagent (0.6M sulfuric acid, 28mM sodium phosphate and 4 mM ammonium molybdate). The tubes were capped and incubated in a boiling water bath at 95^{o}C for 90 min. After the samples had cooled to room temperature, the absorbance of the aqueous solution of each was measured at 695nm against blank in UV spectrophotometer. The blank solution contained 1.0*ml* of reagent solution and the appropriate volume of the same solvent used for the sample and it was incubated under same conditions as rest of the sample. The total antioxidant capacity was expressed as equivalents of ascorbic acid.

$$\% \text{ Scavenging} = \frac{\text{Absorbance of Control-Absorbance of Test Sample}}{\text{Absorbance of Control}} \times 100$$

2.4.5 Assay of in-vivo enzymatic antioxidant activity

Catalase (CAT) Assay: Catalase activity was determined by using hydrogen peroxide (H_2O_2) as a substrate (Chance and Maehly, 1955). Briefly, 0.1*ml* of the supernatant of tissue homogenate was mixed with 2.5*ml* of 50mM phosphate buffer (pH 5.0), 0.4*ml* of 5.9mM H_2O_2 and change in absorbance was recorded at 240nm after one minute of reaction. One unit of CAT activity was defined as an absorbance change of 0.01 as unit/ *ml*.

2.4.6 Biochemical assay

2.4.6.1 Cholesterol: The sera of the rats were analyzed using Randox diagnostic kits to assess the cholesterol level of the rats according to standard protocols as described by the manuals. The cholesterol level was determined after enzymatic hydrolysis and oxidation. The indicator quinoneimine is formed from hydrogen peroxide and 4-aminoantipyrine in the presence of phenol and peroxidase.

2.4.6.2 Triglyceride: The sera of the rats were analyzed using Randox diagnostic kits to assess the triglyceride level of the rats according to standard protocols as described by the manuals.

2.4.7 Statistical analyses

Data from the various studies are presented as mean ± Standard Deviation (SD). The level of significance among the groups was tested using Analysis of Variance (ANOVA) from prism 5 graph pad.

3. Results

3.1.0 Results of phytochemical screening of aqueous ethanolic extract

The phytochemical analysis of aqueous ethanolic leave extract of *P.conophora* showed the presence of Tannins, Phlobatannins, Resins and Flavonoid. However, the presence of alkaloids was not detected.

3.1.1 Phytochemical profile of ellagic acid extract of *Plukenetia conophora* leaves

Phytochemical Profile of Ellagic acid extracted from *P.conophora* showed the presence of the following phytochemicals in the table below:

Table 1: Qualitative determination of ellagic acid extract of *Plukenetia conophora* leaves

	Alkaloid	Flavonoid	Tannin	Phlobatanin	Phenol	Cardiac glycoside	Saponin	Terpernoid	Anthra quinone	Steroid
P.conophora sample	-	-	+	-	+	+	+	+	-	+

Table 2: Quantitative determination of ellagic acid extracts of *Plukenetia conophora* leaves

Phytoconstituent	Absorbance
Tannin mg/100g	47.74±0.080
Phenol mg/100g	36.21±0.070
Cardiac glycoside mg/100g	13.93±0.090
Saponin mg/100g	18.78±0.53
Terpernoid mg/100g	8.675±0.035
Steroid mg/100g	14.21±0.550

Data are expressed as mean ±S.D (n = 2).

This table shows the presence of total quantity of phytochemicals present in ellagic acid extract of *Plukenetia conophora* leaves. Quantitative determination of phytochemicals present in ellagic acid extract showed that tannin had the highest quantity in the extract.

3.1.2 Total antioxidant capacity

The scavenging of the extracts is shown in TABLE 3 and 4. The scavenging ability of ellagic extract is slightly closer to that of ascorbic acid at dose 100µg/ml. Ascorbic acid was taken as 100%. It was observed that the extracts showed a significant of total antioxidant capacity as assessed by reduction of ammonium molybdate when compared to that of ascorbic acid at doses of 10 - 100µg/ml.

Table 3: Total Antioxidant Capacity (TAC) of aqueous ethanolic extract of *Plukenetia conophora* leaves

Concentration	Absorbance	% Scavenging
10µg/ml	0.522±0.010	39.08
20µg/ml	0.733±0.003	21.75
50µg/ml	0.625±0.003	37.12
100µg/ml	0.748±0.016	12.72
ABSCORBIC ACID	0.857±0.007	100

Data are expressed as mean ± SD (n = 3).

Table 4: Total Antioxidant Capacity (TAC) of ellagic acid extract

Concentration	Absorbance	% Scavenging
10µg/ml	0.351±0.004	59.04
20µg/ml	0.248±0.008	71.06
50µg/ml	0.246±0.007	71.29
100µg/ml	0.189±0.010	77.95
ASCORBIC ACID	0.857±0.007	100

Data are expressed as mean ± SD (n = 3).

Table 5: In-Vivo effects of *Plukenetia conophora* on enzymatic antioxidant activity.

Treatment Group	Cat (u/ml)
Control (distilled water only)	3.121±0.024
Postinor	5.431±0.012
Aqueous ethanolic extract	0.317±0.011
Ellagic acid extract	0.591±0.017
Postinor+ extract	0.311±0.013
Postinor+ellagic acid extract	0.424±0.009
Pregnant group (ellagic acid extract) (extract) (distilled water)	0.723±0.016 0.362±0.010 3.294±0.015

Data are expressed as mean ± SD (n = 3) for control groups
Data are expressed as mean ± SD (n = 6) for Postinor group and Test groups

The result was analyzed using mean ± S.D. It was observed that there was significant ($P < 0.001$) decrease in CAT levels in aqueous ethanolic extract and ellagic acid extract when compared with control group and postinor group. Similarly, animals that received aqueous ethanolic extract and postinor followed by treatment with aqueous ethanolic extract showed significant antioxidant activity ($P < 0.001$) by reducing the activity of catalase drastically when compared to control groupand postinor only. In the pregnant group, aqueous ethanolic extract showed statistical reduction ($P < 0.001$) in CAT activity compared to Ellagic acid group and control animals.

Table 6: Testosterone level in test groups and control groups

Treatment Group	Testosterone (ng/ml)
Control (distilled water only)	0.088±0.001
Postinor	0.093±0.002
Aqueous ethanolic extract	0.284±0.001
Ellagic acid extract	0.350±0.001
Postinor+ extract	0.102±0.002
Postinor+ellagic acid extract	0.107±0.003
Pregnant group(ellagic acid extract) (extract) (distilled water)	0.352±0.010 0.277±0.004 0.095±0.001

Data are expressed as mean ± SD (n = 3) for control groups
Data are expressed as mean ± SD (n = 6) for Postinor group and Test groups.

The result shows that there was significant ($P < 0.05$) increase in testosterone in level of aqueous ethanolic extract and ellagic acid extract as compared with the control group.

3.3.0 Result of Cholesterol level in the treatment groups

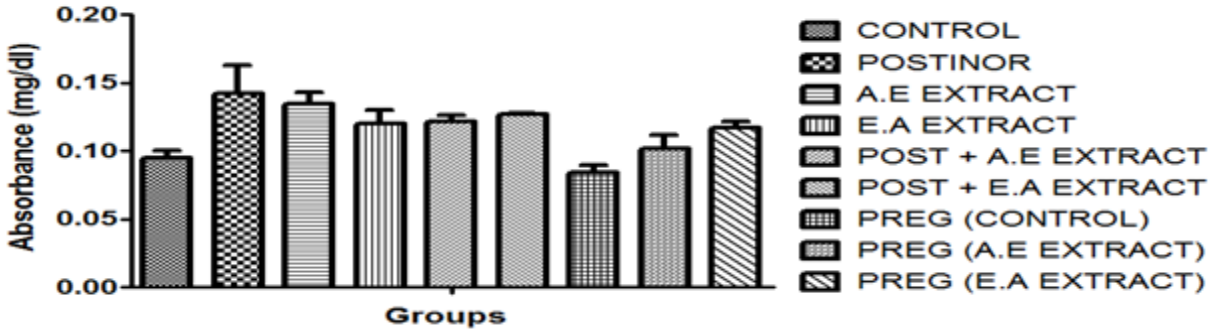

Figure 1: Comparison of Cholesterol levels in different experimental groups treated with *Plukenetia conophora.*

NOTE: A.E Extract ➔Aqueous Ethanolic Extract E.A Extract →Ellagic acid Extract, Data are expressed as mean± SD (n=3) for control groups, Data are expressed as mean± SD (n=6) for Postinor and Test groups

As shown in figure 1, ellagic acid extract group, postinor followed by aqueous ethanolic extract group and postinor followed by ellagic acid extract group had similar cholesterol levels. It was observed that postinor group showed a significant ($P < 0.05$) increase in cholesterol when compared with control group.

3.3.1 Result of Triglyceride in treatment groups

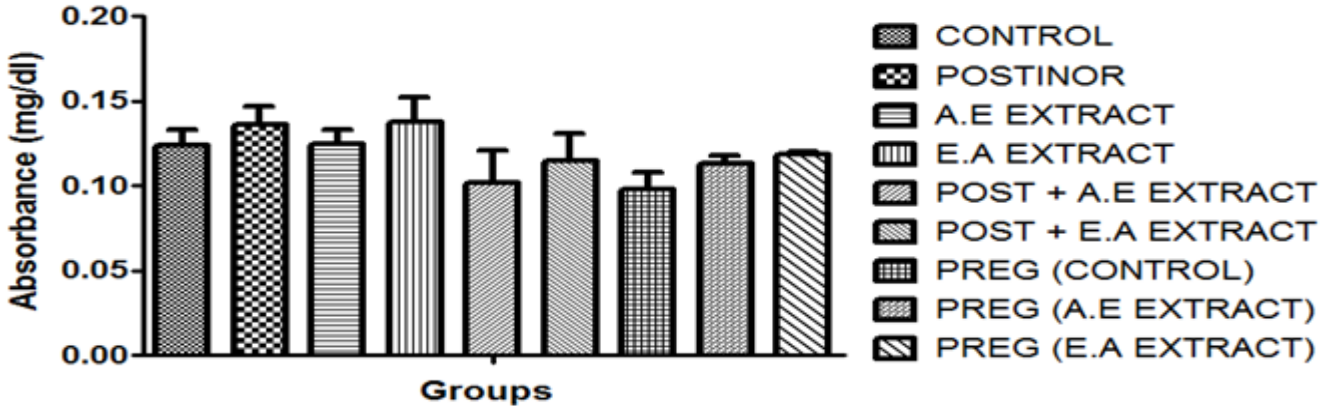

Figure 2: Comparison of Triglyceride levels in different experimental groups treated with *Plukenetia conophora.*

NOTE: A.E Extract ➔Aqueous Ethanolic Extract, E.A Extract →Ellagic acid Extract, Data are expressed as mean± SD (n=3) for control groups, Data are expressed as mean± SD (n=6) for Postinor and Test groups.

As shown in figure 2, postinor group and ellagic extract group showed significant increase ($P < 0.05$) in triglyceride level when compared with control group. In the pregnant group, aqueous ethanolic extract group and ellagic acid extract group showed similar triglyceride level.

4. Discussion

In recent years, the studies on oxidative stress and its adverse effects on human health have become a subject of considerable interest. It is well documented that exposure of organism to exogenous and endogenous factors generate a wide range of reactive oxygen species resulting in antioxidant imbalance (Halliwell et al., 1992).

It is shown from total antioxidant capacity of ellagic acid extract and aqueous ethanolic extract of *P. conophora* assessed by reduction of molybdate which showed high potential to scavenge free radicals when compared to standard ascorbic acid TABLE (3 and 4).

Preliminary phytochemical analysis of both ellagic acid extract and aqueous ethanolic extract revealed the presence of phenolic compounds, tannins, saponins, steroids. The presence of phenolic compound in the extract may act individually or synergistically as free radical terminator. This is believed to be mainly due to their redox properties which play an important role in adsorbing and neutralizing free radical, quenching singlet and triplet oxygen.

It was observed that administration of ellagic acid extract and aqueous ethanolic extract reduced CAT activity significantly ($P < 0.001$) when compared to control and postinor animals. Similarly, the animals that received postinor followed by treatment with the extracts showed significant antioxidant activity ($P < 0.001$) by reducing the activities of catalase drastically when compared to control and postinor group. In the pregnant group, aqueous ethanolic extract group and ellagic acid extract group showed statistical reduction ($P < 0.001$) in catalase activity compared to control animals (TABLE 5).

Following an investigation carried out by Obianime and Uche (2014), on the effects of the aqueous extract of the leaves of *P. conophora* and the effect on the hormonal parameters of female rats. The claims of the use of the leaves of this plant by traditional medicine practioners as a female fertility agent were supported. In their study, they compared the effects of the leaves of *P. conophora*. Using standard testing methods, they observed that the aqueous extracts of *P. conophora* leaves (100mg/ml) caused a statistically significant increase in the level of testosterone of female rats. These effects were dose and time specific. The optimum effect of testosterone level of ellagic acid extract in this study was 0.350 mg/ml ±0.001 and that of aqueous ethanolic extract was 0.284±0.001 at 100mg/ml of *P. conophora* after 30 days treatment.

This study showed that, serum levels of testosterone significantly increase in ellagic acid group and aqueous ethanolic extract group as compared to control. Similarly, control group and postinor only showed statistical high concentration in testosterone level as compared to both ellagic acid extract group and aqueous ethanolic extract group. In the pregnant group aqueous ethanolic extract group and ellagic acid extract group showed significant increase in testosterone level as compared to control group. In postinor group, decrease in serum testosterone level may be due to ability of active component (Levonorgestrel) to inhibit enzyme involved in steroid hormone synthesis.

It was observed that, serum cholesterol level significantly increased in the postinor group as compared to the control group. Postinor + aqueous ethanolic extract group and postinor + ellagic extract group had similar cholesterol level. In the pregnant group, control animal showed significant decrease in cholesterol level ($P < 0.001$) as compared to ellagic acid extract group and aqueous ethanolic extract groups.

Finally, the triglyceride level was significantly decreased in postinor followed by aqueous

ethanolic extract ($P < 0.001$) as compared to the control group. Similarly, postinor group and ellagic acid extract group showed significant increase in triglyceride level ($P < 0.05$) as compared to control groups. It was observed that in the pregnant group, aqueous ethanolic extract group and ellagic acid extract group showed statistical increase in triglyceride level as compared with control group.

5. Conclusion

Based on the in-vitro and in-vivo assay, it can be concluded that the aqueous ethanolic extract and ellagic acid extract of *Plukenetia conophora* possessed good antioxidant activity as evidenced by the free radical scavenging property in augmenting antioxidant defense mechanisms. Overall, the plant is a source of natural antioxidant that may be important in the therapeutic role of free radical mediated disease prevention and health preservation.

The extracts of *Plukenetia conophora* leaves showed good free radical scavenging activity. The aqueous ethanolic of the dried leaves had best antioxidant activity; the broad range of antioxidant of this extract indicates the potential of the plant as a source of natural antioxidants with potential application to reduce oxidative stress and consequent health benefits. The ellagic acid extract of the dried leaves had high amount of tannins. Phenolic compounds found in the plant were likely to be the contributor of antioxidant activity of the leaves extracts.

30 day daily oral administration of ellagic acid extract and aqueous ethanolic extract of *Plukenetia conophora* leaves showed significant increase in cholesterol level, triglyceride level and testosterone level at dose 100mg/kg, this may be due to presence of steroids. It was concluded that *Plukenetia conophora* leaves may be used as female fertility agent.

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