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Phytochemical Constituents and Antioxidant Properties of Methanolic Leaf Extract of *Punica Granatum L.*

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ABSTRACT: *Phytochemical constituent and antioxidant properties of the methanolic leaf extract of Punica granatum was investigated. Extracts of the leaf was subjected to quantitative and qualitative phytochemical screening, In vivo and In vitro scavenging activity against 1, 1-diphenyl-2-picrylhydrazyl (DPPH) and hydrogen peroxide (H_2O_2) were determined. The free radical scavenging activity of DPPH and H_2O_2 have SC_{50} of 1.069±0.29µmol/ml and 1.819±0.29µmol/ml respectively. DPPH actitvity of these extract shows no significant difference when compared with a standard value of Vitamin C is 1.140±0.26µmol/ml whereas H_2O_2 shows significant difference as compared to standared Vitamin C with SC_{50} value of 1.331±0.31µmol/ml. The administration of the extract at 200mg/kg and 400mg/kg body weights of wistar albino rats significantly increased the levels of catalase 39.02±0.72nmol/mg and 39.55±0.62nmol/mg respectively with a negative control of 7.53±1.94nmol/mg as compared to the normal control value of 37.76±0.63nmol/mg whereas lipid peroxidation level decreased significantly at treatment groups 200mg/kg and 400mg/kg with value of 4.96±0.20(nmol/mg)/10^{-6} and 4.35±0.17(nmol/mg)/10^{-6} respectively when compared to the negative control group with a value of 8.21±0.15(nmol/mg)/10^{-6} and normal control value of 4.36±1.67(nmol/mg)/10^{-6}, although there is no significant difference as compared to the normal control groups. These findings suggest possible antioxidant properties of the methanolic leaf extract of Pomegranate.*

KEYWORDS: *Punica granatum,* free radicals scavenging activity, oxidative stress, CCL_4, DPPH, antioxidant activity.

INTRODUCTION

The Pomegranate, botanical name '*Punica granatum',* is a fruit bearing deciduous shrub that contains thousands of health benefits as mentioned three times in the holy Qur'an, thus showing its advantage and privilages as fruits that are beneficial to human beings. In English, it is known as the 'Pomegranate' and '*al- Rumman'* or '*al- Rummanah'* in Arab.(Munirah, 2011.) in older times, the fruit considered in old testament of the Bible, the Jewish Torah, and the Babylonian Talmud as a sacred fruit conferring powers of fertility, abundance, and good luck.(Akhlaghi and Band, 2009).

Pomegranate or Punica granatum, is a small tree growing between 5 and 8 meters (16-26ft) tall, leaves are glossy and lance shaped, it is considered to have originated from the area of southwest Asia and has grown naturally in the entire Mediterranean region and the Caucasus since ancient time, it can be divided into several anatomical compartments including seed, juice, peel, leaf, flower, bark and root with each possessing interesting pharmacological and toxicological activities. (Tanner and Cory, 2009).

Punica granatum L. is a nutrient dense fruit rich in phytochemical compounds, as well as strong antioxidant and antiinflamatory properties. (Miguel *et.al.,* 2010). Recent studies have demonstrated its anti-cancer activity in several human cancers (Adhami and Mukhtar, 2007; Longtin, 2003). Apart from their antioxidant capacity, there have been numerous reports on the in vivo properties of pomegranates, namely on anti-atherosclerotic capacity (Aviram and Dornfeld, 2001; Kaplan and Aviram, 2001), anti-proliferative and pro-apoptotic activities of pomegranate tannin extract (Seeram *et al*., 2005), antiinflammatory activity (Adams *et al*., 2006), as well as chemopreventive and chemotherapeutical potential towards prostate cancer by pomegranate juice (Malik et al., 2005).

The pomegranate contains several major active components including flavonoids.(Jurenka, 2008; Marhari *et.al.*,2014; Suranto and Terbukti, 2011; Arun and Singh, 2012; Yasoubi, 2007.). The benefits of flavonoids are as antibacterial, antiviral, anti-insecticide and anti-inflammatory while the tannin substances as hemostatic, antibacterial, antioxidant and anti-inflammatory.(Prashanth *et.al.*,2001).

The role of antioxidants in human health has prompted some studies in the fields of food science and horticulture to assess fruit and vegetable antioxidants (Kalt *et al*., 1999). They act by scavenging free radicals and by terminating any free radical-induced chain reaction. When an animal is exposed to an external source of free radicals (e.g., as a result of exposure to a toxic chemical) the animals antioxidant defense mechanism steps in and tries to control the rate of deleterious bio-oxidation and molecular damage. The DPPH and H_2O_2 radicals were widely used to investigate the scavenging activity of some natural compounds.

Jeon *et al*., 2003 reported that; Carbon tetrachloride (CCl_4) is an extensively studied xenobiotic that induces lipid peroxidation and toxicity. Carbon tetrachloride (CCl_4) induced Liver cell injury by involves initially the metabolism of CCl_4 to trichloromethyl (CCl_3) free radical by the mixed function oxidase system of the endoplasmic reticulum. The secondary mechanism could involve the generation of toxic products arising directly from CCl_4 metabolism or from peroxidative degeneration of membrane phospholipids, and causes functional and morphological changes in the cell membrane leading to accumulation of lipid-derived oxidants causing liver injury. Moreover, reactive oxygen intermediates (ROIs) generated in the hepatocytes as by-products of CCl_4 metabolism and excess of ROIs, oxidative stress, contribute to cell injury (Kumaravelu *et al*., 1995). CCl_4 induced damage also produces alteration in the antioxidant status of the tissues, which is manifested by abnormal histopathological changes. Several studies have previously

demonstrated that antioxidants prevent CCl_4 toxicity, particularly hepatotoxicty, by inhibiting lipid peroxidation and increasing antioxidant enzyme activities (Kumaravelu *et al*., 1995). Different parts of *Punica granatum L.* were scientifically tested for their medicinal properties by previous workers worldwide, but little information is available on the leaves particularly in Nigeria. This paper therefore determines the Phytochemical constituents and antioxidant properties of Methanolic leaf extract of *Punica Granatum L*.

MATERIALS AND METHODS

Chemicals
Reagents and chemicals used for the experiment are of analar grades. DPPH and Folin Ciocalteau were bought from Sigma Aldrich, United States. All other reagents and chemicals were purchased from various manufacturers.

Plant collection and identification
The fresh leaves of pomegranate were collected from Botanical garden, University of Maiduguri, Borno State, Nigeria and was identified by a plant taxonomist at the Department of Biological Sciences, University of Maiduguri, Nigeria.

Preparation of plant materials
Following collection, the fresh leaves of *Punica granatum Linn* was washed and shade dried. It was then ground into fine powder using mortar and pestle. It was then sieved to remove debris and coarse plant materials. A powder form of the leaves was stored under laboratory condition prior to extraction.

Methanolic extract preparation
About 500g powder of *Punica granatum Linn* leaf was extracted with one liter of 70% methanol using Soxhlet extractor, the extract was then concentrated to dryness at low temperature on a rotary evaporator. The percentage yield of the extract was 13.54%.

Phytochemical screening of Pomegranta methanolic leaf extract (PMLE)
The pomegranate methanol leaf extract (PMLE) was subjected to preliminary phytochemical (qualitative and quantitative) screening/analyses for identification of the various classes of the plant chemical constituents. These analyses were carried out according to the methods described by (Sudiarto and Rifai, 1992).

Phytochemical screening/Analysis of leaf of *Punica granatum*
Phytochemical analysis of the *Punica granatum* leaf was determined using standard analytical methods. Tannins and Flavonoids were determined by the method of Krishnaiah (2009), Alkaloids by Harbone (1998), phenolics by the method of Antolovich (2002) and Ascorbic acid by method as described by Kirk and Sawyer (1998).
The qualitative analysis of *Punica granatum* leaf was determined by the standard method as described by Trease and Evans (2002).

***In vitro* and *In vivo* Antioxidant activity study**

DPPH free radical scavenging activity was determined by the method of Yen and Duh (1994), Hydrogen peroxide scavenging (H_2O_2) assay was as described by (Ruch *et al*., 1989). The *In vivo* antioxidant activity was determined by method of Draper and Hadley (1990), determination of lipid and catalase activity was done as described by Atawodi (2007).

RESULTS

Phytochemical Constituents

Qualitative Phytochemical Analysis of methanolic leaf extract of *Punica granatum L.*

The result of qualitative phytochemical analysis (Table 1) revealed the presence of alkaloids, flavonoids, saponins, carbohydrates, phenol, steroids, and tannins but lacked terpenoids.

Table 1: Qualitative Phytochemical Composition of Methanolic Leaf Extract of *Punica granatum.*

Test	Result
Carbohydrate	+
Tannins	+
Flavanoids	+
Alkaloids	+
Steroids	+
Saponin	+
Terpenoids	-
Phenol	+

Key: + Presence,
- Absent

Quantitative Phytochemical Analysis methanolic leaf extract of *Punica granatum L.*

The result of quantitative phytochemical analysis of the *Punica granatum L*. leaf extract is presented on Table 2. Concentration of phenol 3.70 mg/GAE, Alkaloids 2.03 g, Flavanoids 0.794 g, Tannins 0.616 g and Ascorbic acid VitCmg=1261.3 mg/100g. All these are shown in Table 2.

Table 2: Qualitative Phytochemical Composition of Methanolic Leaf Extract of *Punica granatum.*

Phytochemicals	Concentration (g)
Tannin (g)	0.61 ± 0.01
Flavanoid (g)	0.79 ± 0.01
Alkaloid (g)	2.05 ± 0.01
Phenol (mg/GAE)	3.70 ± 0.17

Values are presented as mean ± SEM,
GAE; Garlic acid equivalent.

Free radical scavenging activity of the methanolic leaf extract of *Punica granatum L.*

The result of this study showed different levels of DPPH free radical scavenging activity with SC_{50} of 1.069±0.29mg/ml as compared to the standard Ascorbic acid 1.140±0.26mg/ml which has no significant difference at $P < 0.05$, and that of Hydrogen Peroxide scavenging activity which is significantly different with SC_{50} of 1.819±0.29mg/ml as compared to the standard 1.331±0.31mg/ml. (Table 3).

Table 3: Free radical scavenging activity (SC_{50}) in µmol/l of methanolic leaf extract of *P.granatum*.

Antioxidant	SC_{50} (DPPH)	SC_{50} (H_2O_2)
Punica granatum (µmol/ml)	1.069±0.29	1.819±0.29*
Vitamin C (µmol/ml)	1.140±0.26	1.331±0.31

Values are presented as mean ± SEM, of four determinations.
Values with different asteric (*) vertically down the column are significantly different. ($P < 0.05$).

Effect of the Methanolic Leaf Extract of *Punica granatum* on *In vivo* Antioxidant parameter (Catalase and Lipid peroxidation).

The Results in Table 4 reveal that Carbon tetrachloride caused a significant reduction in the level of catalase in the negative control group as compared to treatment groups where the plant extract caused a significant increase in the levels while lipid peroxidation level was significantly increased with CCL_4 administration as extract administration significantly decreased the levels close to that of the normal control group.

Table 4: Effect of the Methanolic Leaf Extract of *Punica granatum* on *In vivo* Antioxidant parameter (Catalase and Lipid peroxidation) after 28 days of extract administration.

Group	Catalase (nmol/mg)	Lipid peroxidation (nmol/mg)/10^{-6}
Normal control group	37.76 ± 0.63^{a}	4.36 ± 1.67^{a}
Negative control group (CCL_4)	7.53 ± 1.94^{b}	8.21 ± 0.15^{b}
Positive control group (drug silymarin)	43.72 ± 0.73^{c}	5.88 ± 0.37^{a}
Treatment group (200mg/kg)	39.02 ± 0.72^{a}	4.96 ± 0.20^{a}
Treatment group (400mg/kg)	39.55 ± 0.62^{a}	4.35 ± 0.17^{a}
Extract group (200mg/kg)	45.30 ± 0.51^{dc}	3.96 ± 1.61^{a}
Extract group (400mg/kg)	43.85 ± 1.61^{ec}	5.72 ± 4.15^{a}
Extract group (800mg/kg)	36.31 ± 2.77^{fd}	3.97 ± 1.93^{a}

Values are presented as mean ± SEM, n = 5
Values with different superscript vertically down the group are significantly different ($p < 0.05$)

DISCUSSION

Preliminary phytochemical screening carried out in this study indicated that *Punica granatum* leaf extract contained alkaloids, flavonoids, saponins, carbohydrates, steroids, phenols and tannins, but lacked terpenoids. Pomegranate is an important source of anthocyanins,

hydrolysable tannins punicalagin and punicalin (Afaq *et al.,* 2005), ellagic and gallic acids (Lansky and Newman, 2007) and also contains vitamin C (Turk *et al*., 2008). These phytochemicals are known to perform several general and specific functions in plants, and may exhibit different biochemical and pharmacological actions in different species of animals when ingested. These actions range from cell toxicity to cell protective effects (Trease and Evans, 1996). The quantitative phytochemical analysis of the methanolic leaf extract of *Punica granatum* Linn. showed that concentrations of alkaloids, flavonoids, tannins and phenols, which are known to exhibit pharmacological activities are high. Flavonoids are potent water soluble antioxidants and free radical scavengers that prevent oxidative cell damage, have strong anticancer activity and protect against the different levels of carcinogenesis (Okwu, 2004). The accumulation of these compounds in plant cells have attracted the attention of scientists worldwide and have been the subject of intense investigations. Tannins also react with proteins to produce the typical tannins effect which is important in the treatment of inflammed or ulcerated tissues, burns, wounds, pneumonia and dysentery (Mortan *et al.,* 1987). Plant parts that contain tannins are astringent in nature and have important roles as stable and potent antioxidants (Dharmanda, 2003). Flavonoids have also been found to play a very important role in protection against oxidative stress (Said *et al*., 2008). As shown in Table 2, flavonoids, tannins and alkaloids were found in high concentrations. Thus the high tannins, alkaloids and flavonoids contents of this plant may explain their therapeutic use in herbal medicine**.**

The methanolic leaf extract of *Punica granatum* was found to exert significant antioxidant effect in DPPH and H_2O_2 radical scavenging assay with SC_{50} values of 1.069±0.29µmol/ml and 1.819±0.29µmol/ml respectively as compared to the standard Ascorbic acid (1.140±0.26µmol/ml and 1.331±0.31µmol/ml respectively), this can be related to the work conducted by (Ahmed *et al*., 2011), it was observed that Pomegranate juice (PJ) and Methanolic extract of Pomegranate Pulp (MEPP) had significant effect on some oxidants/antioxidants enzymes of liver and kidney when compared to the control. The therapeutic potentials of plants have been linked with their antioxidant potentials (Akinmalodun *et al*., 2007; Eleazu *et al*., 2011). The decrease in absorbance of DPPH and H_2O_2 caused by the methanolic leaf extract of *Punica granatum* is due to the reaction between antioxidant molecules and radicals, which results in the scavenging of the radical by hydrogen donation.

The *In vivo* antioxidant studies (catalase and Lipid peroxidation) revealed a decreased value of 7.53± 1.94 in the negative control group as compared to normal control (37.76± 0.63). The administration of the extract significantly increased the levels to a level similar to that of the normal control group at two different doses of 200mg and 400mg per kg body weights with values of 39.02±0.72 and 39.55±0.62 respectively for catalase. This shows that the methanolic leaf extract of *Punica granatum* has a significant effect on the catalase activity. There is also a significant increase in the lipid peroxidation with a value of 8.21±0.15 in the negative control group as compared to the normal control (4.36±1.67), administration of the extract therefore decreased the levels to a level closer to that of the normal control values at two different doses of 200mg and 400mg per kg body weights with values of 4.96±0.20 and 4.35±0.17 respectively at $p<0.05$. Several researchers have observed that the activities and expression of antioxidant

enzymes such as catalase, glutathione peroxidase and SOD drops drastically in the liver and kidney of animals exposed to an external source of free radicals compared to control animals (Khalaf *et al.,* 2009, Hamed *et al.,* 2016, Mahmoud *et al.,* 2015, Hafez *et al.,* 2014, Zhang *et al.,* 2013, El-Nekeety *et al.,* 2014). This may be explained by the fact that these radicals may inhibit (non-specifically) the synthesis as well as the activity of antioxidant enzymes in the liver and kidney at the DNA level and at the protein level respectively (Birben *et al.,* 2012, Weber *et al.,* 2003, Dalton *et al.,* 1999, Siu and Draper, 1982).

CONCLUSIONS

Punica granatum methanolic leaf extract has antioxidant property and hence it may be used for therapeutic purposes.

Recommendation

The mechanism of action as well as isolation of compound responsible for this effects should be studied.

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