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In vitro Free Radical Scavenging and Analgelsic Activities of *Parinari Kerstingii* Leaves

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Abstract: The study was aimed to investigate in vitro free radical scavenging activity and Analgelsic Activities of Parinari Kerstingii Leaves Extract.

The in vitro free radical scavenging activity of Parinari kerstingii leaf extract was studied through DPPH radical scavenging assay, Ferric reducing antioxidant power ability (FRAP), Nitric oxide (NO) radical scavenging assay and total antioxidant capacity, using ascorbic acid and gallic acid as standard. The analgesic activity of the plant extract was also evaluated using acetic-acid-induced mice writhing and hot plate tests. The phytochemical constituents of the extract were also determined. The extract showed high DPPH radical scavenging activity as shown by the high percentage inhibition (86 %) and lowest value of EC50 (79.23 µg/mL), indicating the strong hydrogen-donating ability. The extract manifested significant reducing power (0.745) which exceeded even that of gallic acid (0.710) at a concentration of 1000 µg/mL. At a maximum concentration of 1000 µg/mL, the extract showed 97.2 % inhibition of nitric oxide and also exhibited a scavenging effect of 78.9 % on total antioxidant capacity. The results also showed that the extract (100-400 mg/kg) significantly ($P<0.05$) and dose-dependently prolonged the pain reaction time in hot-plate pain model, and reduced acetic acid-induced writhing.

Phytochemical analysis revealed the presence of tannins, saponins, reducing sugars, phenols, soluble carbohydrates, alkaloids, terpenoids, steroids, hydrogen cyanide glycosides and flavonoids. These findings indicate that the leaf extract of Parinari kerstingiimay play an important role in chemical protection from oxidative damage by possessing endogenous antioxidants, such as phenolics, tannins and flavonoids.

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Key words: Antioxidant, free radical, analgesic activity, *Parinari kerstingii*

Introduction

Antioxidants are defined as molecules which can safely interact with free radicals and terminate the chain reaction before vital molecules are damaged (Nidhi and Gauray, 2012). They prevent or inhibit free radical formation and defend mechanism of the organism against the pathologies associated to the attack of free radicals (Pisoschi and Negulescu, 2011). Reactive oxygen species (ROS) are different forms of activated oxygen which consist of free radicals such as hydroxyl radical (OH.), superoxide radical (O_2^-), Nitric oxide (NO^-), peroxyl (RO_2^-), lipid peroxyl (LOO^-) radicals and non-free radical species such as hydrogen peroxide (H_2O_2), singlet oxygen (O_2^{-1}), ozone (O_3), lipid peroxide (LOOH), (Yildrin and Mavi, 2000; Gulcin *et al.*,2004). ROS are produced by all aerobic organisms and can easily react with most biological molecules including proteins, lipids, lipoproteins and DNA, leading to an imbalance between the systemic manifestation of reactive oxygen species and a biological system's ability to readily detoxify or repair the resulting damage (Halliwell, 2007). ROS levels can increase markedly during times of environmental stress thereby generating oxidative stress, which could lead to many pathophysiological disorders such as arthritis, diabetes, inflammation, cancer and genotoxicity (Gulcin *et al*, 2002b). One of the most important approaches to the primary prevention or treatment of these diseases may be minimizing oxidative damage, since antioxidants may interrupt an oxidizing chain reaction or stop the free radical formation. The use of traditional medicine is widespread and plants are potential source of natural antioxidants that might serve as leads for the development of novel drugs (Conforti *et al.*, 2009). Plant-derived antioxidants have been shown to function as singlet and triplet oxygen quenchers, free radical scavengers, peroxide decomposers, enzyme inhibitors and synergists (Palanisamy and Ling, 2012). Antioxidants such as the vitamins A, C and E, beta carotene, flavonoids, mineral selenium have potential to prevent oxidative damage and demonstrated their potent antioxidant activities both *in vitro* and *in vivo* (Isaac *et al*., 2008; Pandey and Rizvi, 2009; Hamid *et al*., 2010). This has led to a great research interest in naturally occurring antioxidants, especially in plants, as an alternative solution to health problems. In this present study, we want to evaluate the antioxidant and analgesic activities of the methanol extract of the leaf of *Parinari Kerstingii.*

Materials and Methods

Plant collection and preparation

Fresh leaves *Parinari kerstingii* were obtained from Aba-Achara town of Nsukka Local Government Area, Enugu state. It was identified by Mr A. Ozioko of International Centre for Ethnomedicine and Drug Development, Nsukka. The voucher specimen

number is INTERCEDD 0615 was deposited at the same centre. The leaves were air-dried and milled with a mechanical grinder. The ground plant (5000 g) was macerated in methanol for 24 hrs, filtered with a Whatman No. 1 filter paper and the filtrate concentrated using a rotary evaporator (IKA, Germany) at an optimum temperature of 40–50°C.

Phytochemical Screening

The plant extract was screened for the following phytochemicals: saponins, tannins, flavonoids, alkaloids, terpenoids, glycosides, steroids, using standard procedures of Trease and Evans, (1983).

Antioxidant activity

DPPH radical scavenging assay: The DPPH radical scavenging activity of the extracts was investigated using the method described by Brand-Williams *et al.* (1995). One milliliters of the crude extract (0.62-1 mg/mL CH_3OH) were added to 1ml of 0.06 mM DPPH in methanol. The mixture was allowed to stand for 30 minutes in the dark. Absorbance was measured at 517 nm against CH_3OH as blank. Ascorbic acid was used as standard.

Ferric reducing antioxidant power ability (FRAP)

Ferric reducing antioxidant power activity (FRAP) was determined by method of Benzie and Strain (1996). FRAP assay measures spectrophotometrically the absorbance of a blue colour generated by a reduction from the ferric 2, 4, 6-tripyridyl-s-triazine complex [Fe (III)-$(TPTZ)^2]^{3+}$ to ferrous complex [Fe (II)-$(TPTZ)^2]^{2+}$ by antioxidants in acidic solution at 700 nm. In the FRAP method, the antioxidant activity is quantified using ascorbic acid as a reference. Two milliliters of the crude extract (0.3-1 mg/mL) in normal saline were mixed with 2 mL of 0.2 M phosphate buffer (pH 6.6) and 2 mL of 1 % potassium ferricyanide. After 20 minutes incubation at 50 ^{0}C, 2 mL of 10 % trichloroacetic acid (TCA) was added. After 5-10 minutes the mixture was centrifuged (1000 x g; 10 min) and 2 mL supernatant was collected and diluted with 2 mL distilled water. The sample was mixed with 0.4 mL of 0.1 % $FeCl_3$ and the absorbance was measured at 700 nm. The activity was expressed as gallic acid equivalent/gram

Nitric oxide (NO) radical scavenging assay

Nitric oxide radical scavenging activity was measured spectrophotometrically according to the method described by Marcocci *et al.* (1994). When sodium nitroprusside was mixed with aqueous solution at physiological pH, suddenly it generates nitric oxide, which reacts with oxygen to produce nitrite ions that can be estimated using Greiss reagent. Nitric oxide scavengers compete with oxygen leading to reduced production of nitrite ions. The nitric oxide scavenging activity of the extract was conducted based on Greiss assay method. Sodium nitroprusside (2.0 mL of 10 mM) in phosphate buffer (pH 7.4) and 5.0 mL of phosphate buffer was mixed with 0.5 mL of different concentrations (0.62-1.0 $mgmL^{-1}$) of the plant extract and incubated at room temperature for 150 minutes.

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After the incubation period, 2 mL of the incubated solution was added to 2 mL of Greiss reagent (1 % sulphanilamide, 0.1 % α-napthyl-ethylene diamine dihydrochloride and 3 % phosphoric acid) and incubated at room temperature for 30 minutes. The absorbance of the pink chromophore formed by the diazotization of nitrite with α-napthyl-ethylene diamine dihydrochloride was measured at 540 nm. Ascorbic acid was used as positive control and results was expressed as percentage inhibition of nitric oxide. All determinations were performed in triplicates. The percentage inhibition was calculated using the formula below:

$$\%\ Inhibition = 100\ X\ \frac{Ac-At}{Ac}$$

Where: Ac = Absorbance of the control, At = Absorbance of the test drug/extract

Total antioxidant capacity (TAC)

Total antioxidant capacity of the extract was estimated using the phosphomolybdate method as reported by Saeed *et al.* (2012). The assay is based on the reduction of Mo (VI) to Mo (V) by the extract and subsequent formation of green phosphate/Mo (V) complex at acid ρH. A 0.3 mL extract was combined with 3 mL of reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). The absorbance of the reaction mixture was measured at 765 nm using a spectrophotometer (Jenway 6025) against blank after cooling to room temperature. Methanol (0.3 mL) in the place of extract was used as the blank. The antioxidant activity is expressed as the number of gram equivalent of ascorbic acid. The calibration curve was prepared by mixing ascorbic (1000, 500, 250, 125, 62.5 and 31.25 µg/mL) with methanol.

Analgesic Assays

Acetic acid-induced writhing test in mice

The method described by Koster *et a*l., (1959), was employed to assess the analgesic activity of the methanol leaf extract of *parinari kersting*ii. A total of 25 mice were divided into 5 groups of 5 mice each. The mice were treated with distilled water (5 mL/kg), *parinari kersting*ii extract (100, 200 and 400 mg/kg) or indomethacin (10 mg/kg) orally. 30 minutes post-treatment, mice in all groups were treated with acetic acid 10 ml/kg of 0.6 % v/v intraperitoneally. The number of writhes was counted 5 minutes after acetic acid injection for a period of 25 minutes. Percentage inhibition of writhing was calculated using the formula:

Hot plate method

The effect *Parinari kerstingii* extract of on hot plate analgesia was determined by method of (Turner, 1965). Twenty-five mice were fasted for 6 hours, divided into five groups of five animals each and were administered distilled water (5 mL/kg); aspirin (100 mg/kg); plant extract (100, 200, 400 mg/kg). Each mouse was gently placed on the hot plate maintained at 50 ^{0}C and the time required by the mouse to lick the

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paw or jump were taken as the response. The percentage protection was calculated.

Results

Phytochemical screening

The plant extract was qualitatively screened for common phytochemicals. The results are shown in Table 1. The results revealed the presence of the phytochemicals that were screened for.

Table 1 **Phytochemical content of Methanol extract of *Parinari kerstingii* leaves.**

Phytochemical	**Bioavailability**
Alkaloids	++
Saponins	++
Steroids	+
Terpenoids	++
Glycosides	++
Tannins	++
Flavonoids	+++
Reducing Sugar	++
Phenols	+
Hydrogen Cyanide	+
Soluble Carbohydrate	+

Key: + Mild, ++ Moderate, +++ Abundance

Antioxidantactivity

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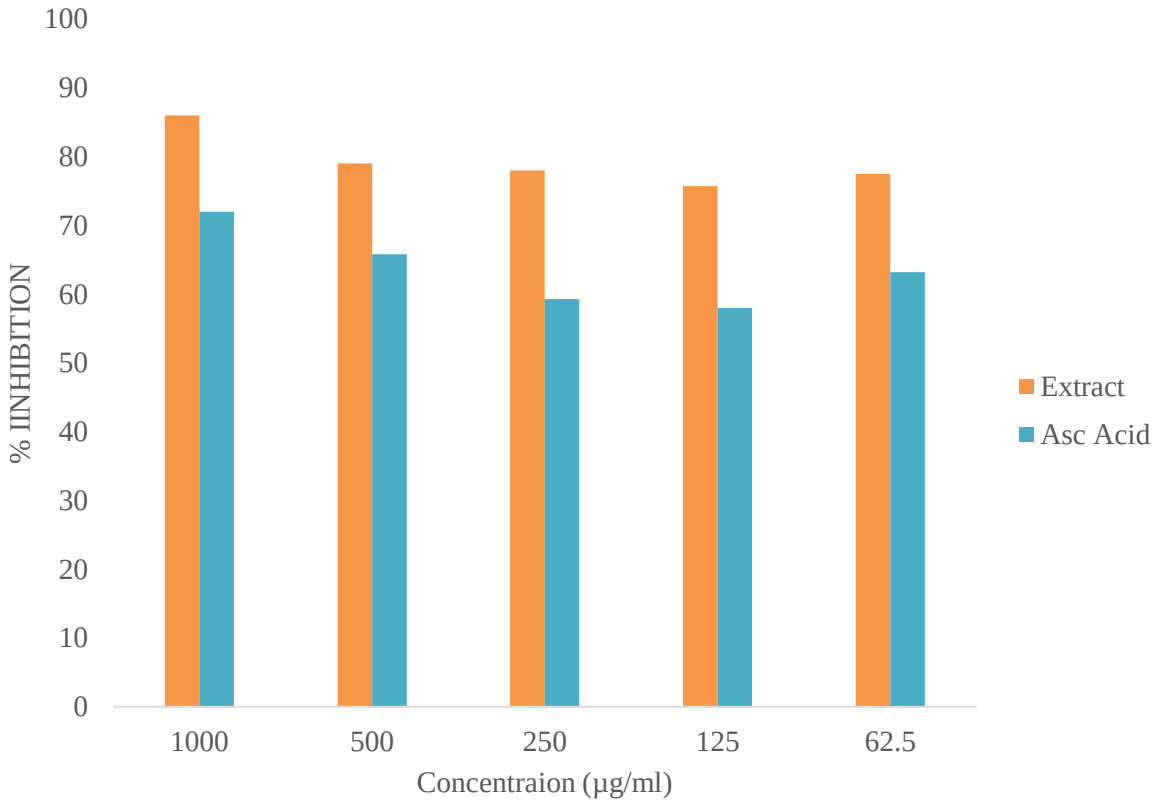

Fig 1: Effect of plant extract on DPPH radical scavenging activity

Table 2 Ferric Reducing Antioxidant power of *Parinari kerstingii* leaves extract

SAMPLE	**Absorbance (700nm)**		**GAE/g**
Conc (mg/ml)			
	Extract	**Gallic Acid**	
0.0312	0.67±.050 [c]	0.34±.007[a]	1.96
0.0625	0.70±.001 [c]	0.39±.022[a]	1.79

0.125	0.70±.019 [c]	0.42±.053[a]	1.67
0.25	0.69±.003 [c]	0.54±.084[b]	1.28
0.5	0.69±.009 [c]	0.64±.142[b]	1.08
1.0	0.75±.071 [c]	0.71±.084[c]	1.04

Values represents mean ±standard deviation of triplicate samples. Mean values, down the column, with different letters as superscripts are considered significant at ($p<0.05$)

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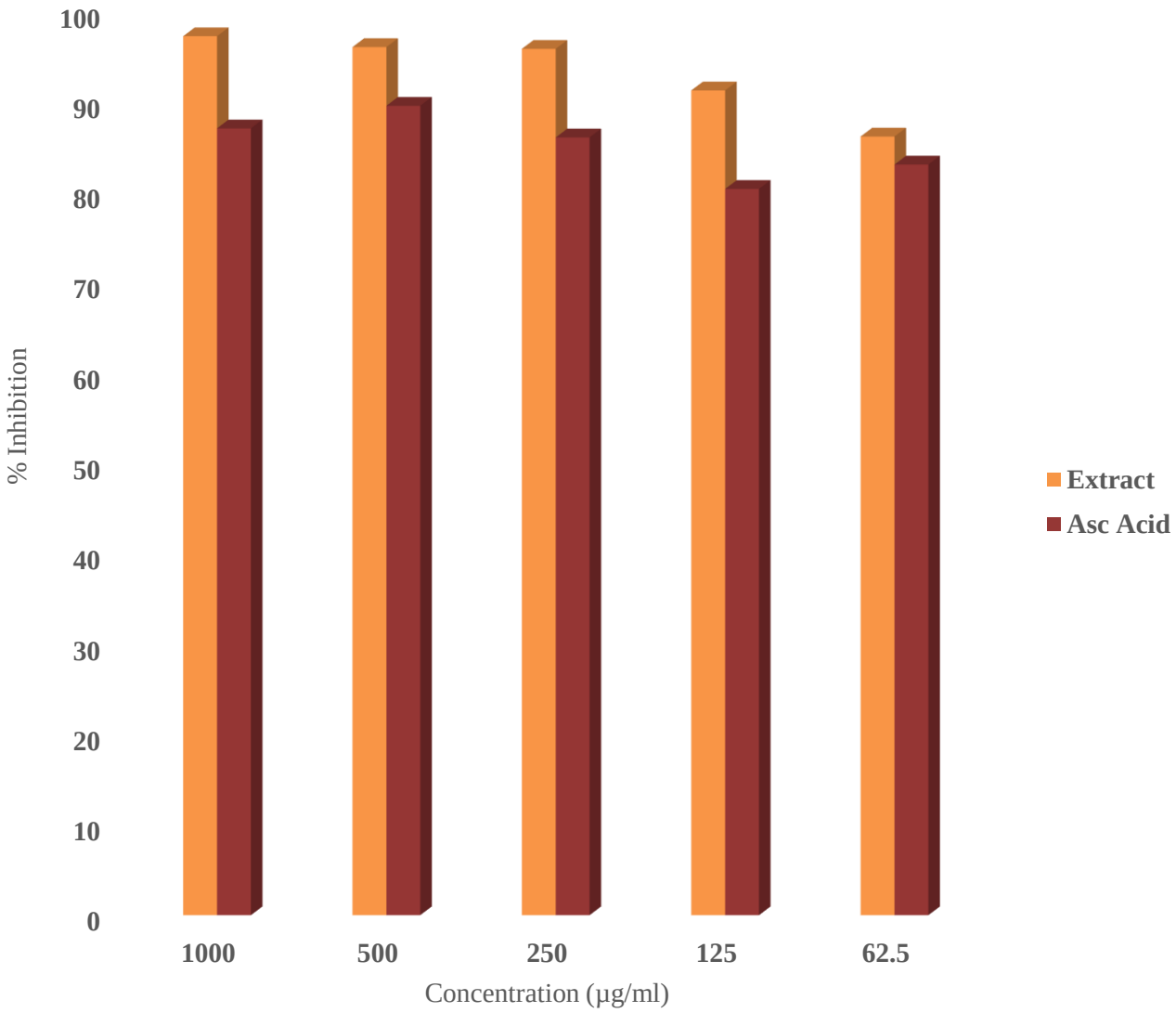

Fig 2: Effect of plant extract on NO radical scavenging activity

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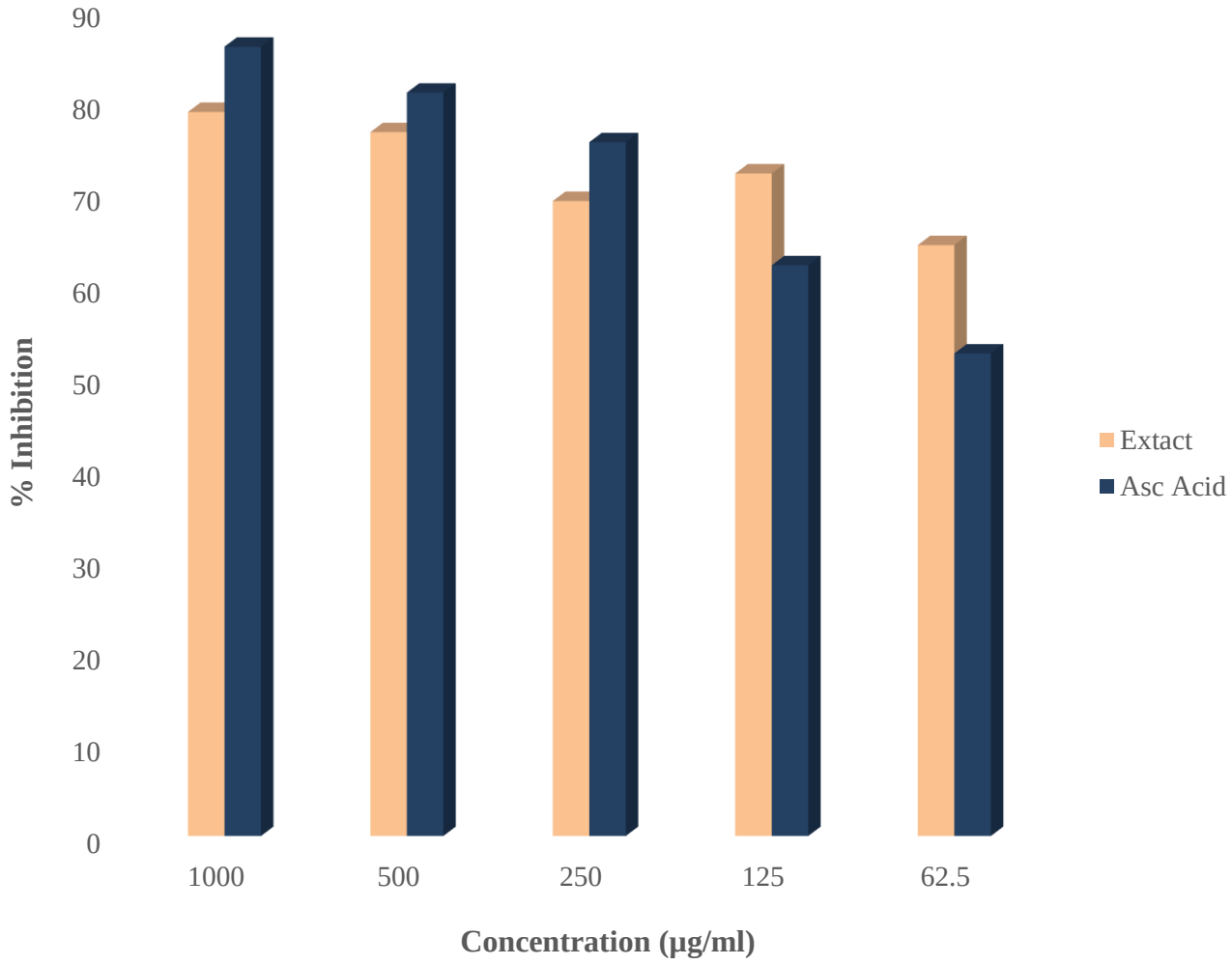

Fig 3: Effect of plant extract on TAC

Analgesic activity

Table 3: Effect of Methanol leaves Extracts of *Parinari kerstingii* on Acetic-acid-induced mice writhing test

Treatment Group	Number of Writhing	Percentage Inhibition

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Control	92.50±6.156[d]	-
Aspirin (ASA)	0.00±0.00[a]	100
100mg/kg	31.36±2.408[c]	69
200mg/kg	22.20±7.463[b]	76
400mg/kg	19.80±4.147[b]	78.5

Values represents mean ±standard deviation of triplicate samples. Mean values, down the column, with different letters as superscripts are considered significant at $p < 0.05$

Table 4: Effect of Methanol leaves Extracts of *Parinari kerstingii* on thermally induced pain stimulus

Treatment Groups	**Reaction latency time (sec)**	
	15min	**15min**
Control	2.4±0.8[a]	2.6±0.8[a]
Aspirin, ASA (100mg/kg)	2.8±0.8[a]	7.7±1.9[b]
100mg/kg	3.61±1.8[a]	11.6±2.5[c]
200mg/kg	4.2±1.9[a]	12.8±1.3[c]
400mg/kg	5.8±2.1[b]	16.8±4.8[d]

Values represents mean ±standard deviation of triplicate samples. Mean values, down the column, with different letters as superscripts are considered significant at $p < 0.05$

Discussion

The phytochemical analysis of the plant extract was found to be rich in total phenolic compounds, flavonoids, terpenoids and tannins as shown in table 1 and these compounds were reported to have significant antioxidant properties (Dong, 2003). Total phenolics constitute one of the major groups of compounds acting as primary antioxidants or free

radical terminators hence a reasonable amount was detected in the extract. Flavonoids are the most widespread group of natural compounds and probably the most important natural phenolics. Total phenolics and flavonoids possess a broad spectrum of chemical and biological activities including radical scavenging properties. The medicinal effects of plants are often attributed to the antioxidant activity of phytochemical constituents mainly phenolics, flavonoid (Miliauskas *et al*., 2004). It is claimed that phenolic compounds are powerful chain breaking antioxidants (Shahidi, 1992) and the scavenging activity of phenolic group is due to its hydroxyl group. (Hateno *et al*., 1987)

The extract showed a rapid discolouration of the purple DPPH to light yellow, suggesting a high hydrogen-donating ability in the presence of DPPH stable radical and this could be as a result of phenolic compounds, terpenoids and flavonoids which are likely to contribute to the radical scavenging effect of this plant extract. This is consistent with the report of Volluri *et al*., (2011) which stated that phenolics, triterpenoids and flavonoids possess many of the properties of an efficient free radical scavenger, and their hydrogen donor capacity probably accounts in large part for the antioxidant activities of this extract. The DPPH radical scavenging activity was recorded in terms of % Inhibition as shown in fig. 1. It was observed from fig.1 that 0.06 mg/mL extract has minimum DPPH scavenging activity (75.5 % ± 0.77 %) and 1 mg/mL concentration of the extract has maximum DPPH scavenging activity (86.0 ± 3.47 %) among individual concentration of the plant extract. The Results obtained were comparative to standards Ascorbic acid used, with minimum and maximum DPPH scavenging activity of 0.06 mg/mL (58 %) and 1 mg/mL (72 %) respectively. Higher % Inhibition indicates better scavenging activity or antioxidant potential. 1 mg/mL concentration of the extract has shown better DPPH scavenging activity as compared to individual concentration of the plant extract. The results obtained were statistically significant with $p<0.05$. The total antioxidant capacity of the *Parinari kerstingii* extract was equally recorded in terms of % Inhibition as shown in fig. 3. It was observed from Fig.3 that 0.06mg/ml extract has minimum TAC (52.6 % ± 0.77 %) and 1 mg/mL concentration of the extract has maximum TAC (86.0 ± 3.47 %) among individual concentration of the plant extract. The Results obtained were comparable to standard Ascorbic acid used, with minimum and maximum TAC activity of 0.06 mg/mL (64.4 %) and 1 mg/mL (78.6 %). Higher % Inhibition indicates better scavenging activity or antioxidant potential. 1 mg/mL concentration of the extract has shown better TAC scavenging activity as compared to individual concentration of the plant extract. The results obtained were statistically significant with $p<0.05$. Our results were consistent with the observation that *R. agrestis* contain radical-scavenging agents that could directly react with and quench stable DPPH radicals of which possesses flavonoids as the most important constituents in the leaves (Bitis *et al*., 2010). The effective concentration (EC_{50}) sufficient to elicit 50 % of a maximum effect

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estimated in 100 % was calculated to be approximately 79.23 µg/mL and 97.02 µg/mL for the DPPH radical scavenging effect of the extract and ascorbic acid with regression coefficients (r^2) of 0.79 and 0.91, respectively. This shows a good antioxidant activity by the methanol extract of *P. kerstingii*.

As observed from Table 2, 0.03 mg/mL extract has minimum reducing power (0.66 ± 0.015) and 1 mg/mL concentration of the extract has maximum reducing power (0.75 ± 0.086) amongst individual concentration of the plant extract. Higher absorbance indicates more reducing power. 1mg/ml concentration of the extract has shown more reducing power in comparison to individual concentration of the plant extract. The results obtained were statistically significant with $p< 0.05$. The nitric oxide radical scavenging activity was also recorded in terms of % Inhibition as shown in fig. 2. It was observed from Fig.2 that 0.06 mg/mL extract has minimum NO scavenging activity (59.8 % ± 0.77 %) and 1mg/ml concentration of the extract has maximum NO scavenging activity (97.2± 3.47 %) among individual concentration of the plant extract. The Results obtained were comparative to standards Ascorbic acid used, with minimum and maximum NO scavenging activity of 0.06 mg/mL (80.3 %) and 1 mg/mL (91 %). Higher % Inhibition indicates better scavenging activity or antioxidant potential. 1 mg/mL concentration of the extract has shown better NO scavenging activity as compared to individual concentration of the plant extract. The results obtained were statistically significant with $p<0.05$. Reducing power is associated with antioxidant activity and may serve as a significant reflection of the antioxidant activity (Oktay *et al*., 2003). It is reported that antioxidant activity and reducing power is one and the same (Hammam, 2008). Compounds with reducing power indicate that they are electron donors, and can reduce the oxidized intermediates of lipid peroxidation processes, so that they can act as primary and secondary antioxidants (Yen and Chen, 1995). Some studies have also reported that the reducing capacity of a compound may serve as a significant indicator of its potential antioxidant activity (Jeong *et al*., 2004; Jamuna *et al*., 2012). As shown in fig 2, the reductive abilities of the extract were concentration dependent, its activity increases with increasing concentration. Therefore, it is speculated that the presence of flavonoids in this species might contribute a big way for the effectiveness of reducing power.

NO is a free radical with a single unpaired electron; and one of the signaling molecule that participates in almost every cellular and organ functions of the body (Thenmozhi. *et al.,* 2014). Nitric oxide is generated based on the principle that sodium nitroprusside in aqueous solution at physiological pH spontaneously generate nitric oxide (NO**.**) which interacts with oxygen to produce nitrite ions (NO_2^-), that can be estimated. Scavengers of nitric oxide compete with oxygen, leading to reduced production of nitrite ions (Rajesh and Natvar, 2011). In this study, *Parinari kerstingii* extract inhibited nitric oxide generation in a

concentration dependent manner with higher scavenging activity (97.2 %) at concentration of 1000 µg/mL which compared well to that of ascorbic acid (87 %). The nitric oxide molecules may be directly scavenged by flavonoids and this is coherent with the study which states that several flavonoids, including quercetin, result in a reduction in ischemia-reperfusion injury by interfering with inducible nitric-oxide synthase activity (Shoskes, 1998). When flavonoids were used as antioxidants, free radicals are scavenged and therefore can no longer react with nitric oxide, resulting in less damage (Shutenko *et al*., 1999). Therefore, it has been speculated that phytochemicals such as flavonoids and tannins present in these extracts might play a vital role in scavenging of NO radicals. Total antioxidant capacity model is based on the reduction of Mo (VI) to Mo (V) by the extract and subsequent formation of green phosphate/Mo (V) complex at acidic ρH. The extracts demonstrated electron donating capacity and thus they may act as radical chain terminators, transforming reactive free radical species into stable non-reactive products (Dorman 2003).

The results of this study also revealed that the methanol leaf extract of *P. kerstingii* possess both peripheral and central analgesic properties. Acetic acid-induced pain model is usually used for the evaluation of mild peripheral analgesic (Hassan *et al*, 2008) because it is very sensitive and able to detect anti-nociceptive effects of compounds at dose levels that may appear inactive in other methods. A dose-dependent reduction in the number of writhes was produced by oral doses (100, 200 and 400 mg/kg) of the extract (Tab 3). ASA produced the most percentage inhibitory effect. Treatment groups exhibited a significant ($P \leq 0.05$) effect when compared to that of the control group. In the hot plate method (fig. 4), the number of writhes decreased significantly in ASA treated groups as compared to the extract treated groups. Effect of *Parinari kerstingii* was observed to be dose dependent, but the peak activity was recorded 60min after treatment. However, the plant extract at 400 mg/kg significantly increased in latency period as compared to the standard drug, ASA. Also, ASA showed a significant ($P \leq 0.05$) increase in the 60min post-drug treatment when compared to the control group. Different doses of extract significantly ($P \leq 0.05$) showed increased in latency period at 60 minutes post-drug treatment when compared to the control group. The analgesic effect of the plant extract could either be as a result of its action on visceral receptor sensitive to acetic acid, to the inhibition of the algogenic substance or inhibition of transmission of painful messages at the central level (Hosseinzadeh and Younesi, 2002; Hosoi, 1999). The abdominal injection of acetic acid in an experimental animal has been attributed to the release of arachidonic acid, which results in the synthesis of prostaglandin through the cyclooxygenase (COX) enzyme (Farias *et al* 2011). The sensory nerve endings that sense pain is very sensitive to prostaglandin and when prostaglandin is released, the sensory nerve respond to it through prostaglandin E_2 (PGE_2) receptor by taking up and

transmitting pain and injury through the nervous system to the brain and cause visceral writhing stimuli in mice (Da Silva *et al* 2011). On the other hand, thermal painful stimuli are selective for the evaluation of centrally acting analgesic drugs, but not peripherally (Chau, 1989). These results suggest that the extract may possess NSAID- like and opiod-like analgesic activities, mediated through both the peripheral and central mechanisms.

Conclusion

The methanol extract of *Parinari kerstingii* leaves exhibits high antioxidant, free radical scavenging activities and has reducing power. These *in vitro* assays indicate that this plant extract is a significant source of natural antioxidant, which might be helpful in preventing the progress of various oxidative stresses. It has also shown to have a significant analgesic property and could serve as a natural remedy in the management of pains.

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