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Pharma, Drug Discovery, Toxicology & Medicine

Highlights

- Paracetamol – Induced Liver Extract of Lactuca teraxacifolia
- Antioxidant Activity of Aqueous Properties of Cocos nucifera Juice

Discovering Thoughts, Inventing Future

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Antioxidant Activity of Aqueous Extract of *Blighia sapida* Stem Bark in Alloxan-induced Diabetic Rats

By Amira, Philip. O & Oloyede, Hussein O. B

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Abstract- *Blighia sapida* is a plant belonging to the family of sapindaceae. In this study we aimed to evaluate the in vivo antioxidant activities of aqueous extract of *Blighia sapida* stem bark in alloxan-induced diabetic rats. Administration of the extract at 100mg/kg body weight significantly ($P<0.05$) increased the activities of antioxidant enzymes catalase, glutathione peroxidase and superoxide dismutase in the kidney and pancreas tissues of diabetic rats. Also the concentration of reduced glutathione increased in the kidney and pancreas tissues of the diabetic rats while the levels of malondialdehyde and protein carbonyl generally decreased in the kidney and pancreas tissues of alloxan-induced diabetic rats during the course of the experiment. These are indications of antioxidant properties of the stem bark of *Blighia sapida* with 100mg/kg body weight of the aqueous extract showing good antioxidant activities by comparing favourably well with metformin, a standard antidiabetic drug.

Keywords: *blighia sapida, diabetes, antioxidant enzymes, biomolecules.*

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Antioxidant Activity of Aqueous Extract of *Blighia sapida* Stem Bark in Alloxan-induced Diabetic Rats

Amira, Philip. O [α] & Oloyede, Hussein O. B [σ]

Abstract- ***Blighia sapida*** **is a plant belonging to the family of sapindaceae. In this study we aimed to evaluate the *in vivo* antioxidant activities of aqueous extract of *Blighia sapida* stem bark in alloxan-induced diabetic rats. Administration of the extract at 100mg/kg body weight significantly ($P<0.05$) increased the activities of antioxidant enzymes catalase, glutathione peroxidase and superoxide dismutase in the kidney and pancreas tissues of diabetic rats. Also the concentration of reduced glutathione increased in the kidney and pancreas tissues of the diabetic rats while the levels of malondialdehyde and protein carbonyl generally decreased in the kidney and pancreas tissues of alloxan-induced diabetic rats during the course of the experiment. These are indications of antioxidant properties of the stem bark of *Blighia sapida* with 100mg/kg body weight of the aqueous extract showing good antioxidant activities by comparing favourably well with metformin, a standard antidiabetic drug.**

Keywords: blighia sapida, diabetes, antioxidant enzymes, biomolecules.

I. Introduction

Diabetes mellitus is a group of metabolic disease caused by a defect in insulin production, insulin action or both. Type 1 diabetes is caused by a lack of insulin due to the destruction of insulin-producing β – cells in the pancreas. Type 2 diabetes, the most common form of diabetes is caused by a combination of factors, including insulin resistance, a condition in which the body's muscle, fat and liver cells do not use insulin effectively.

Diabetes mellitus is a multifactorial disease, which is characterized by hyperglycemia (Ugochukwu *et al*., 2003), lipoprotein abnormalities (Scoppola *et al*., 2001), raised basal metabolic rates (Okwu *et al*., 2006), defect in reactive oxygen species scavenging enzymes and altered intermediary metabolism of major food substances (Unwin *et al*., 2001).

Hyperglycemia causes many of the health problem associated with diabetes, including eye, kidney, heart disease and nerve conditions. Hypoglycemic agents have been used in the management of diabetes mellitus (DM).

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The World Health Organization (WHO) in its 2014 release reported that the prevalence of diabetes has reached epidemic proportions. In 2014 the global prevalence of diabetes was estimated to be 9% among adults aged 18+ years. In 2012, an estimated 1.5 million deaths were directly caused by diabetes. More than 80% of diabetes deaths occur in low- and middle-income countries (WHO, 2014).

Diabetes mellitus is associated with an increase in reactive oxygen species (ROS) generation by mononuclear cells and an increased oxidative load resulting in oxidative damage to lipids, proteins and DNA (Marfella *et al*., 1995; Giugliano *et al*., 1997; Paoliso and Giugliano, 1996).

Chronic hyperglycemia and subsequent augmentation of reactive oxygen species (ROS) deteriorate β-cell functions and increase insulin resistance which leads to the aggravation of type 2 diabetes (Kaneto *et al.*, 2010). It has been shown that ROS are produced in various tissues under diabetic conditions (Baynes and Thorpe, 1999).

Chronic hyperglycemia is a cause of impairment of insulin biosynthesis and secretion. This process is called β-cell glucose toxicity which is often observed under diabetic conditions. β-cells are rather vulnerable to ROS due to the relatively low expression of antioxidant enzymes such as catalase and glutathione peroxidase. Therefore it is likely that ROS are involved in β–cell deterioration found in diabetes (Evans *et al.*, 2003). It is also known that lipotoxicity is also involved in the deterioration of β-cell function found in type 2 diabetes (Kaneto *et al.*, 2010).

Blighia sapida is a plant belonging to the family of Sapindaceae. It is commonly known as ackee. In Nigeria, it is called Gwanja Kusa (Hausa), Isin (Yoruba) and Okpu (Igbo) (Aderinola *et al.*, 2007). Most of the earlier studies on *Blighia sapida* have been on the nutritional qualities of the root (Abolaji *et al*, 2007) and the leaves as a dry season feed resource for West African dwarf goats in the Northern savanna zone of Nigeria (Aderinola *et al*, 2007). The repellant potential of the fruit part components against stored-product insect pests (Khan and Gumbs, 2003) as well as neutropenia and thrombocytopenia effects of the aqueous and lipid extracts of the unripe fruit have been investigated in

mice (Gardiner *et al*, 1996). More recently, the physicochemical properties of the oil from the fruit of the species and toxicological evaluation of the oil – based diet in Wister rats have been investigated (Oladiji *et al*, 2009).

However, the scanty information on the antioxidant activity of extract of *Blighia sapida* stem bark prompted this study. Stem bark is an important component of African traditional medicine as herbal medicine is still the main source of health care for the majority of Africans and in particular, Nigerians. There has been increasing demand for the use of plant products with anti-diabetic activity. The high cost, availability, uncertainty of use during pregnancy and undesirable side effects of synthetic drugs or drugs from other animal sources are some of the factors leading to a strong preference for hypoglycemic drugs of plants origin.

II. Materials and Methods

1. *Chemicals*: All chemicals used were of analytical grade and items are products of BDH and Sigma Chemical Ltd., UK and Accu-chek ® Advantage, Roche Diagnostic, Germany.
2. *Animals*: Male albino rats (*Ratus norvegicus*) weighing between 100g and 120g were used for the experiment. The rats were bred in the animal holding of the Department of Anatomy and Cell Biology, Obafemi Awolowo University, Ile-Ife, were maintained on standard rat pellets (Ladokun feeds, Ibadan, Nigeria), and were given water *ad libitum*.
3. *Sourcing for the Tree Bark of Blighia sapida:* A sizeable quantity of the tree bark of *Blighia sapida* was obtained from the compound of the Federal Polytechnic, Ado Ekiti, Nigeria.
4. *Identification of Plant*: The fruits and leaves of *Blighia sapida* plant were obtained from the compound of the Federal polytechnic, Ado Ekiti, Ekiti State, Nigeria and were used for the purpose of authentication of the identity of the plant at the Herbarium unit of the Department of Plant Biology, University of Ilorin, Ilorin, Nigeria. The voucher number of identification is UIH624.
5. *Processing of sample and preparation of extract*: The sample obtained was air-dried at room temperature for fifty-six (56) days until a constant weight was obtained. The air-dried tree bark of *Blighia sapida* was pulverized. 100g of the pulverized sample was extracted with 800ml of distilled water for seventy-two (72) hours in an extractor. The aqueous extract was obtained by filtering with Whatman filter paper and subsequently freeze-dried in Armfield freeze-drier for ten (10) days. The residue obtained was weighed and the percentage yield was calculated.
6. *Induction of experimental diabetes mellitus*: After an overnight fasting, rats were induced by intraperitoneal administration of alloxan monohydrate at a dose of 120mg/kg body weight. Alloxan monohydrate was freshly dissolved in distilled water and maintained on ice prior to use. Four days after the administration, the animals were fasted for 16 hours and blood glucose levels were determined in mg/dl using a digital glucometer (Accu-chek ®, advantage, Roche, Diagnostic, Germany) and animals which had basal glycemia levels of 125mg/dl were used in the experiment.
7. *Experimental Design*: Randomized Complete Block Design (RCBD) method was used. Eighty male albino rats were grouped as follows:

Group 1: Control group administered with distilled water orally.

Group 2: The alloxan-induced diabetic group left untreated

Group 3: The alloxan-induced diabetic group treated with oral administration of distilled water extract of *Blighia sapida* at 100mg/1000g body weight

Group 4: The alloxan-induced diabetic group treated with oral administration of Metformin hydrochloride at 21.4mg/1000g body weight.

All the animals were fed with vital finisher made up of maize and soya bean mainly. The administration of the extracts as written above was carried out every 24 hours for 21 days.

Analysis of the various parameters as stated was carried out weekly after diabetes detection for three weeks.

8. *Repeated administration of the aqueous extract of Blighia sapida stem bark in control and diabetic groups*: The fasting blood glucose levels of all groups were measured and then the extract dissolved in distilled water. The solution of the extract was administered to one of the diabetic groups orally at 100mg/kg body weight once a day for twenty-one (21) days.The diabetic control and untreated (without alloxan induction). Five animals each were sacrificed from each of the four groups by chloroform anaesthesia and the pancreas and kidney obtained from them. The pancreas and kidney so obtained were stored in phosphate buffer (0.1M, pH = 7.0) maintained below -20^{0}C until required for analysis.
9. *In vivo Antioxidant Assay:* Pancreas and kidney tissues were homogenized with cold 1.5% KCl to make a 10% homogenate.

   Determination of the activity of Catalase (CAT): Catalase activity was determined in the lysate using Aebi's method (Aebi, 1984).

   Determination of the activity of Superoxide dismutase (SOD): This method is well described by Mccord and Fridovich (1969).

Determination of the activity of Glutathione Peroxidase (GPx): Glutathione peroxidase (GPx) was measured by the method described by Rotruck *et al.*, (1973).

Determination of reduced glutathione (GSH): Reduced glutathione (GSH) was measured by the method of Beutler and Kelly (1963). The amount of GSH is expressed in mg/100g tissue.

Determination of Malondialdehyde (MDA): Total amount of lipid peroxidation products present in the samples was estimated by the thiobarbituric acid (TBA) method which measures the malondialdehyde (MDA) reactive products according to the method of Ohkawa *et al*., (1979).

Determination of Protein Carbonyl Content: The protein carbonyl content was assayed according to a previous method of Levine *et al* (1990).

Determination of Protein: Protein determination was carried out according to the method of Lowry *et al.,* (1951) as described by Holme and Peck, (1998).

10. *Statistical Analysis*: Data were expressed as mean ± S.E.M. of five replicates and subjected to one-way analysis of variance (ANOVA) followed by Duncan's multiple range test to determine significant differences in all the parameters. Values were considered statistically significant at $P<0.05$.

III. Results

1. *Catalase activity:* Specific activity of catalase was found to increase ($P<0.05$) in kidney and pancreas following administration of aqueous extract of *Blighia sapida* stem bark while the administration of metformin, a standard antidiabetic drug increased the specific activity of catalase in kidney and pancreas till the fourteenth day of the experiment (Table 4). The specific activity of catalase was found to reduce in the kidney and pancreas of untreated, diabetic animals.

Table 4: Specific activity of catalase in kidney and pancreas of diabetic albino rats following administration of Aqueous extract of *Blighia sapida* stem bark

Tissue	Group of animal	Specific activity of catalase (Units/mg protein) ($x10^{-2}$)			
		0 day	7th day	14th day	21st day
Kidney	Untreated control	148.44± 2.41[a]	149.35± 2.10[a]	148.52± 3.01[a]	148.24± 2.52[a]
	Diabetic control	128.20± 13.60[b]	113.85± 14.00[b]	103.98± 13.20[b]	82.65± 13.40[b]
	Diabetic + Aqueous extract	128.20± 13.60[b]	104.71± 2.88[c]	230.22± 1.86[c]	279.55± 0.01[c]
	Diabetic + Metformin	123.50± 0.08[b]	71.40± 0.08[d]	112.00± 0.23[b]	86.00± 0.50[b]
Pancreas	Untreated control	94.13± 2.53[a]	94.13± 2.53[a]	98.32± 3.12[a]	92.01± 2.51[a]
	Diabetic control	25.06± 4.90[b]	23.76± 3.10[b]	19.85± 2.00[b]	12.58± 1.80[b]
	Diabetic + Aqueous extract	25.06± 4.90[b]	11.51± 2.30[c]	21.08± 2.90[b]	120.83± 3.32[a]
	Diabetic + Metformin	24.90± 0.28[b]	35.80± 0.31[d]	52.20± 0.36[c]	20.00± 0.39[c]

Values are mean of five determinations ± S.E.M. Values with different superscript in the row and column differ significantly ($p<0.05$)

2. *Glutathione peroxidase (GPx) activity:* A significant increase ($P< 0.05$) was observed in the specific activity of glutathione peroxidase in the pancreas of the diabetic rats after an initial reduction, following administration of aqueous extract of *Blighia sapida* stem bark. On the other hand, the specific activity of glutathione peroxidase in the kidney of diabetic rats did not increase but significantly reduced ($P < 0.05$) during the course of the experiment, a result similar to the one obtained for the untreated diabetic rats (Table 5).

3. *Superoxide dismutase (SOD) activity*: Table 6 shows an initial significant increase ($P < 0.05$) in the specific activity of superoxide dismutase followed by a slight reduction toward the end of the experiment in the kidney of the diabetic rats following administration of aqueous extract of *Blighia sapida* stem bark. However, the specific activity of superoxide dismutase in the pancreas of the diabetic rats significantly increased ($P < 0.05$) during the course of the experiment. A significant increase ($P < 0.05$) in the specific activity of superoxide dismutase was observed in both the kidney and pancreas of diabetic rats following administration of metformin, a standard antidiabetic drug.

Table 5: Specific activity of Glutathione peroxidase (GPx) in kidney and pancreas of diabetic albino rats following administration of Aqueous extract of *Blighia sapida* stem bark

Tissue	Group of animal	Specific activity of Glutathione peroxidase (Units/mg protein) (XI0·5)			
		0 day	7th day	14th day	21st day
Kidney	Untreated control	61.20± 3.97[a]	60.20± 3.02[a]	61.20± 3.96[a]	60.00± 4.02[a]
	Diabetic control	54.l0± 1.38[b]	50.10± 1.21[b]	43.10± l.31[b]	51.30± 1.2l[b]
	Diabetic + Aqueous extract	54.l0± 1.31[b]	73.70± 0.47[c]	39.70± 0.73[b]	22.30± 0..28[c]
	Diabetic + Metformin	3l.00± 4.35[c]	29.00± 4.22[d]	26.20± 4.83[c]	63.70± 6.05[a]
Pancreas	Untreated control	86.80± 3.0l[a]	81.30± 2.96[a]	86.90± 3.00[a]	90.00± 3.02[a]
	Diabetic control	68.90± 1.14[b]	61.20± 1.02[b]	49.30± 1.11[b]	40.30± 2.0l[b]
	Diabetic + Aqueous extract	68.90± 1.l4[b]	46.70± 1.32[c]	34.90± 0.11[c]	45.20± 0.59[b]
	Diabetic + Metformin	46.90± 2.96[c]	17.00± 3.51[d]	2l.00± 3.34[d]	15.00± 2.44[c]

Values are mean of five determinations ± S.E.M. Values with different superscript in the row and column differ significantly (p<0.05)

Table 6: Specific activity of superoxide dismutase (SOD) in kidney and pancreas of diabetic albino rats following administration of Aqueous extract of *Blighia sapida* stem bark

Tissue	Group of animal	Specific activity of superoxide dismutase (SOD (Units/mg protein) (x10·3)			
		0 day	7th day	14th day	21st day
Kidney	Untreated control	19.35± 10.06[a]	20.16± 1. 26[a]	19.56± 1.01[a]	19.35± 1.16[a]
	Diabetic control	28.05± 1.23[b]	26.52± 0.96[a]	28.95± 1.24[a]	19.98± 1.02[a]
	Diabetic + Aqueous extract	28.05± 1.23[b]	193.36± 3.04[b]	116.07± 2.34[b]	109.22± 1.81[b]
	Diabetic + Metformin	32.00± 1.40[b]	84.00± 1.31[c]	123.00± 1.32[b]	382.00± 0.50[c]
Pancreas	Untreated control	17.32± 5.48[a]	17.32± 5.48[a]	18.51± 1.05[a]	19.32± 5.46[a]
	Diabetic control	16.72± 6.28[a]	10.27± 2.15[b]	9.81± 0.53[b]	6.01± 0.23[b]
	Diabetic + Aqueous extract	16.72± 6.28[a]	166.91± 12.21[c]	116.53± 16.28[c]	245.27± 52.55[c]
	Diabetic + Metformin	24.23± 1.90[b]	66.00± 1.72[d]	77.00± 7.50[d]	98.00± 7.40[d]

Values are mean of five determinations ± S.E.M. Values with different superscript in the row and column differ significantly (p<0.05)

4. *Reduced glutathione:* Table 7 shows the effect of administration of aqueous extract of *Blighia sapida* stem bark on concentration of reduced glutathione (GSH) in kidney and pancreas of diabetic rats. There was a significant ($P < 0.05$) increase in the level of reduced glutathione, a potent antioxidant, in the kidney and pancreas of diabetic rats after an initial reduction, following the administration of aqueous extract of *Blighia sapida* stem bark.
5. *Malondialdehyde:* A significant reduction ($P < 0.05$) in the level of malondialdehyde (MDA) was noticed in the kidney and pancreas of diabetic rats following the administration of aqueous extract of *Blighia sapida* stem bark (Table 8). On the other hand, the administration of metformin, a standard antidiabetic drug, did not reduce the concentration of malondialdehyde, Instead the level of malondialdehyde increased throughout the course of the experiment in the kidney and pancreas tissues of the diabetic rats treated with metformin, a similar result obtained in the group of untreated diabetic rats.
6. *Protein carbonyl:* Table 9 shows a significant reduction ($P < 0.05$) towards the end of the experiment after an initial increase, in the level of protein carbonyl in the kidney tissues of diabetic rats following the administration of aqueous extract of *Blighia sapida* stem bark. On the other hand, the level of protein carbonyl in the tissue of the pancreas of diabetic rats treated with aqueous extract of *Blighia sapida* stem bark did not follow any particular pattern. Also, while the level of protein carbonyl in the tissues of pancreas in diabetic rats treated with metformin ultimately reduced ($P < 0.05$) those in the tissues of kidney of diabetic rats treated with metformin did not follow a definite pattern.

Table 7: Concentration of reduced glutathione (GSH) in kidney and pancreas of diabetic albino rats following administration of Aqueous extract of *Blighia sapida* stem bark

Tissue	Group of animal	Concentration of Glutathione (GSH) (mM/mg tissue)			
		0 day	7th day	14th day	21st day
Kidney	Untreated control	0.99± 0.17[a]	0.97± 0.10[a]	0.99± 0.18[a]	1.08± 0.20[a]
	Diabetic control,	1.82± 0.35[b]	1.84± 0.25[b]	1.l7± 0.19[a]	0.98± 0.51[a]
	Diabetic + Aqueous extract	1.82± 0.35[b]	1.22± 0.12[a]	1.69± 0.14[b]	3.26± 0.16[b]
	Diabetic + Metformin	2.36± 0.06[c]	1.09± 0.06[a]	1.53± 0.06[b]	1.62± 0.08[c]
Pancreas	Untreated control	0.4l± 0.17[a]	0.41± 0.13[a]	0.59± 0.10[a]	0.50± 0.09[a]
	Diabetic control	1.04± 0.14[b]	1.16± 0.52[b]	1.04± 0.26[b]	1.01± 0.19[b]
	Diabetic + Aqueous extract	1.04± 0.41[b]	1.17± 0.06[b]	1.07± 0.09[b]	1.70± 0.11[c]
	Diabetic + Metformin	1.06± 0.09[b]	1.44± 0.09[b]	l.45± 0.04[c]	2.97± 0.06[d]

Values are mean of five determinations ± S.E.M. Values with different superscript in the row and column differ significantly ($p<0.05$)

Table 8: Concentration of malondialdehyde (MDA) in kidney and pancreas of diabetic albino rats following administration of Aqueous extract of *Blighia sapida stem* bark

Tissue	Group of animal	Concentration of malondialdehyde (MDA) (mmol/mg tissue) (xl0·5)			
		0 day	7th day	14th day	21st day
Kidney	Untreated control	.1186.68± 0.14[a]	1193.00± 0.10[a]	1l86.88± 0.11[a]	1099.80± 0.10[a]
	Diabetic control,	1426.50± 0.12[b]	1522.20± 0.10[b]	1700.90± 0.12[b]	lS92.30± 0.12[b]
	Diabetic + Aqueous extract	1426.50± 0.12[b]	1364.38± 0.99[b]	1351.30± 0.03[a]	1052.30± 0.08[a]
	Diabetic + Metformin	1390.00± 0.06[b]	2570.00± 0.51[c]	3570.00± 0.25[c]	9750.00± 0.22[c]
Pancreas	Untreated control	1278.59± 0.22[a]	1288.60± 0.10[a]	1392.60± 0.20[a]	1278.59± 0.22[a]
	Diabetic control	2227.10± 0.08[b]	2723.50± 0.09[b]	2965.20± 0.09[b]	3435.20± 0.09[b]
	Diabetic + Aqueous extract	2227.10± 0.08[b]	1625.82± 0.07[c]	1227.10± 0.28[a]	1106.20± 0.06[a]
	Diabetic + Metformin	2010.00± 0.17[b]	2300.00± 0.17[b]	2700.00± 0.07[b]	2800.00± 0.31[c]

Values are mean of five determinations ± S.E.M. Values with different superscript in the row and column differ significantly ($p<0.05$)

Table 9: Concentration of protein carbonyl in kidney and pancreas of diabetic albino rats following administration of Aqueous extract of *Blighia sapida* stem bark

Tissue	Group of animal	Concentration of protein carbonyl (micromol carbonyl/mg tissue)			
		0 day	7th day	14th day	21st day
Kidney	Untreated control	0.77± 7.96 E-07[a]	0.82± 8.22 E-07[a]	0.79± 7.83 E-07[a]	0.75± 6.99 E-07[a]
	Diabetic control	1.l8± 8.60 E-07[b]	1.28± 7.79 E-07[b]	1.63± 8.24 E-07[b]	2.03± 7.87 E-07[b]
	Diabetic + Aqueous extract	1.18± 8.60 E-07[b]	1.50± 4.50 E-07[b]	0.82± 5.82 E-07[a]	0.86± 7.39 E-07[a]
	Diabetic + Metformin	1.42± 1. 16 E-05[c]	2.80± l.58E-05[c]	1.66± l.56E-05[c]	3.21± 1.45E-05[c]
Pancreas	Untreated control	1.33± 7.26 E-07[a]	1.30± 6.99 E-07[a]	1.31± 8.22 E-07[a]	1.34± 7.10 E-07[a]
	Diabetic control	0.90± 5.14 E-07[b]	1.52± 6.01 E-07[b]	2.08± 5.02 E-07[b]	2.94± 5.01 E-07[b]
	Diabetic + Aqueous extract	0.90± 5.14 E-07[b]	1.35± 5.63 E-07[a]	0.75± 3.03 E-07[a]	1.46± 6.11 E-07[a]
	Diabetic + Metformin	1.23± 5. 16 E-06[b]	0.94± 8.22E-06[c]	1.83± 8.88E-06[c]	1.32± 2.74E-05[c]

Values are mean of five determinations ± S.E.M. Values with different superscript in the row and column differ significantly ($p<0.05$)

IV. Discussion

Diabetes mellitus is associated with an increase in reactive oxygen species (ROS) generation by mononuclear cells and an increased oxidative load resulting in oxidative damage to lipids, proteins and DNA. Acute hyperglycemia has been shown to result in an increase in blood pressure, which is prevented by antioxidants, this suggests that acute hyperglycemia probably causes increased generation of ROS. Chronic hyperglycemia and subsequent augmentation of reactive oxygen species (ROS) deteriorate β-cell functions and increase insulin resistance which leads to the aggravation of type 2 diabetes (Kaneto *et al*, 2010).

It has been shown that ROS are produced in various tissues under diabetic conditions (Baynes and Thorpe, 1999). There are several sources of ROS in cell such as nonenzymatic glucosylation reaction, the electron transport chain in mitochondria, and membrane-bound NADPH oxidase (Browlee, 2001; Harrison *et al*, 2003, Mohazzab *et al*, 1994). Chronic hyperglycemia is a cause of impairment of insulin biosynthesis and secretion. This process is called β-cell glucose toxicity which is often observed under diabetic conditions. In diabetic state, hyperglycemia and subsequent production of ROS decrease insulin gene expression and finally bring about apoptosis. In addition, ROS are induced and involved in the β-cell glucose toxicity. β -cells are rather vulnerable to ROS due to the relatively low expression of antioxidant enzymes such as catalase, glutathione peroxidase and superoxide dismutase. Therefore it is likely that ROS are involved in β-cell deterioration found in diabetes (Evans *et al*, 2003). The potential mechanism of oxidative stress includes the reduction of antioxidant defense. In this study, the levels of catalase, glutathione peroxidase and superoxide dismutase activities in the tissues of kidney and pancreas of diabetic group were significantly reduced and treatment with *Blighia sapida* stem bark aqueous extract improved the catalase (CAT), glutathione peroxidase (GPx) and superoxide dismutase (SOD) activities not only on acute experiments but also after 21 days of treatment. Decreased levels of CAT, GPx and SOD in the diabetic state may be due to the inactivation caused by reactive oxygen species. In treated groups, the increased CAT specific activity could be due to higher production of H_2O_2. It is possible that CAT activity which in turn would protect SOD inactivation by H_2O_2 causes an increase in SOD activity. Increase in SOD activity would protect GPx and CAT against inactivation by superoxide anion (Blum and Fridovich, 1985). An increase in the level of reduced glutathione could be due to it been spared as a result of the protection offered by superoxide dismutase to glutathione peroxidase.

The increase in free radicals in diabetic condition is suggested to be due to the increased lipid peroxidation and the damage to antioxidant defense systems. Protein glycation and glucose autoxidation can generate free radicals that catalyze the lipid peroxidation (Altan *et al*, 2006).

In particular O_2^- and $^{\cdot}OH$ induce various injuries to the surrounding organs and play a vital role in some clinical disorders. Therefore, removal of O_2^- and $^{\cdot}OH$ is the most effective defense of the living body against disease (Lin *et al*, 1995). Any compound, natural or synthetic, with antioxidant activity might totally or partially alleviate this damage. In this study, direct effects of aqueous extract of *Blighia sapida* stem bark on malondialdehyde (MDA) levels in diabetic group were found to be higher ($P < 0.05$) than those in control group, indicating increased free radical generation. Treatment of diabetes with the aqueous extract of *Blighia sapida* stem bark caused a general reduction in the MDA levels in kidney and pancreas after 21 days of treatment.

Direct effects on protein carbonyl levels in diabetic group were found to be higher than those in control group ($P < 0.05$), indicating increased free radical generation via production of various kinds of glycated proteins such as glycosylated hemoglobin, albumin and lens. Treatment of diabetes with the aqueous extract of *Blighia sapida* stem bark caused a reduction in the level of protein carbonyl in kidney and pancreas within 21 days of administration.

V. Conclusion

A major finding of this study is that *Blighia sapida* stem bark aqueous extract generally caused a significant increase in the activities of catalase, glutathione peroxidase and superoxide dismutase in the kidney and pancreas of diabetic rats during 21 days of treatment. It is also noticed that aqueous extract of *Blighia sapida* stem bark extract possess the capability of inhibiting or reducing both lipid and protein peroxidation in diabetes.

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In *Vitro* Cytotoxic Screening of Combination of Honey Bee Venom and Ethanol Extract of Zingiberaceae and *in Vivo* Anticancer Evaluation of them against DLA and EAC Bearing Mice

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Abstract- In spite of the fact that substantial advancement have been made in the remedy and control of cancer progression, remarkable inadequacy for improvement remain. Natural therapy can diminish adverse effect of chemotherapy. Currently over 60% of the drugs are derived in one or other way from natural source including plant, marine organism and micro-organism. The present investigation was concerned with pharmacological potential of honey bee venom and ethanol extract of Zingiber officinale Roscoe towards anticancer activity. The cytotoxic potency of combination of honey bee venom and ethanol extract of Zingiber officinale Roscoe was evaluated on cultured cancer cells Hep-2 by sulphoradamine B assay (IC_{50}=53 µg/ml) and moreover, it was invested by brine shrimp lethality assay (IC_{50}=48.31 µg/ml), tryphan blue exclusion assay (IC_{50}=37.49 µg/ml), and MTT assay. MTT assay exhibited that combination of honey bee venom and ethanol extract of Zingiber officinale Roscoe has more cytotoxicity potency towards human breast adenocarcinoma cells (MCF-7) than normal Chinese hamster lung fibroblast cells (V79).

Keywords: honey bee venom, zingiber officinale roscoe, cytotoxic potency, human breast adenocarcinoma cells.

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In *Vitro* Cytotoxic Screening of Combination of Honey Bee Venom and Ethanol Extract of Zingiberaceae and *in Vivo* Anticancer Evaluation of them against DLA and EAC Bearing Mice

Cytotoxic Screening and Anticancer Activity of Honey Bee Venom and Ethanol Extract of *Zingiber Officinale Roscoe*

Dr. Somayeh Afsah Vakili [α], Ajay George [σ] & Syed Fayazuddin [ρ]

Abstract- In spite of the fact that substantial advancement have been made in the remedy and control of cancer progression, remarkable inadequacy for improvement remain. Natural therapy can diminish adverse effect of chemotherapy. Currently over 60% of the drugs are derived in one or other way from natural source including plant, marine organism and micro-organism. The present investigation was concerned with pharmacological potential of honey bee venom and ethanol extract of *Zingiber officinale Roscoe* towards anticancer activity. The cytotoxic potency of combination of honey bee venom and ethanol extract of *Zingiber officinale Roscoe* was evaluated on cultured cancer cells Hep-2 by sulphoradamine B assay (IC_{50}=53 µg/ml) and moreover, it was invested by brine shrimp lethality assay (IC_{50}=48.31 µg/ml), tryphan blue exclusion assay (IC_{50}=37.49 µg/ml), and MTT assay. MTT assay exhibited that combination of honey bee venom and ethanol extract of *Zingiber officinale Roscoe* has more cytotoxicity potency towards human breast adenocarcinoma cells (MCF-7) than normal Chinese hamster lung fibroblast cells (V79). In EAC ascites model significantly ascending of life span with restoring of haematological parameters and additionally, in DLA solid tumour model crucial decline in the tumour weight and tumour volume was observed as compared to control.

Keywords: honey bee venom, zingiber officinale roscoe, cytotoxic potency, human breast adenocarcinoma cells.

I. Introducton

One of the governing causes of global mortality is cancer (Lopez et al., 2006). World Health Organization (WHO) reported that there were 8.2 million deaths in 2012 and it is estimated up to 13.1 million deaths in 2030 (Ferlay et al., 2008). In the United States, one in four deaths is ascribed to cancer (Jemal et al., 2007). Cancer cells are susceptible to chemotherapy by reason of losing normal function and uncontrolled proliferating of cells. Nevertheless, some of the anticancer drugs have carcinogenicity themselves such as alkylating agents and antracycline antibiotics (Sharma and Sharma, 2007). Natural products have tremendous potential to issue newest medicines since their natural chemicals may supply chemoprotective potential versus cancer. Bee venom contains major components that include histamine catecholamines, polyamines, melittin, and phospholipase A2. Melittin represents about 50-70% of all antimicrobial peptides present in bee venom. Some antimicrobial peptides isolated from insects display a wide range of biological activities including melittin, cecropin related peptides and the magainins which have been shown to exhibit antitumor activity for cells derived from mammalian and human tumours. It is also one of the most potent inhibitors of calmodulin activity and a potent inhibitor of cell growth and clonogenicity (Orsolic et al., 2009). *Zingiber officinale Roscoe* belongs to family *Zingiberaceae* commonly called as ginger (Radhakrishnan, 2014). Ginger contains the volatile compounds such as alpha-zingiberene, beta-sesquiphellandrene, alpha-farnesene, beta-bisabolene, alpha-curcumene, which are mostly consisted of sesquiterpene hydrocarbons and the non-volatile pungent compounds are mainly oleoresin (gingerol, shogaol), phenol (zingerone, gingeol). Ginger also possesses anthelmintic, anti-bacterial and anti-viral activities. Moreover, ginger was found to be active against inflammatory, allergic, degenerative, cardiovascular and metabolic disorders and anticancer activity (Poltronieri et al., 2014). The current investigation was undertaken to anticipate the anticancer potential of combination of honey bee venom and ethanol extract of *Zingiber officinale Roscoe*.

II. Materials and Methods

a) Plant material and Preparation of extract

The rhizomes of *Zingiber officinale Roscoe* (*Zingiberaceae*) were collected from Mysore District, Karnataka State, India and authenticated by Green

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Chem of India, Bangalore, Karnataka, India; a voucher specimen (MZO-GR-101) was conserved for future references. The rhizomes were dried and converted into fine powder using an electrical blender. Fine powder (100 g) was homogenized in ethanol (95%; 500 mL) and left in a conical flask at room temperature for 3 days. The mixture was filtered through a fine muslin cloth and a filter paper (Whatman No. 1). The extract became concentrated by using the Eyela rotary evaporator. The percentage yield of ethanol extract of *Zingiber officinale Roscoe* was 12%.

b) Bee venom

Lyophilized whole bee venom was purchased from New Technique Laboratory Ltd (Georgia). Bee venom was reconstituted in distilled water to obtain the desirable concentrations for *invitro* and *invivo* study and centrifuged at 12,000 rpm for 10 minutes to remove insoluble materials.

c) Chemicals

3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), Fetal bovine serum (FBS), Sulphoradamine B (SRB), Minimum essential medium (MEM) and Trypsin were purchased from Sigma-Aldrich Comany, Bangalore, India; 96 well plates, T flasks (T-25 cm^2), were purchased from Tarsons, Kolkata, India. All other chemicals were analytical grade.

d) Cell lines

Human breast adenocarcinoma cells (MCF-7), Normal V79 cells (Chinese hamster lung fibroblast) and HEp-2 (Human epithelial carcinoma) cells procured from National centre for cell sciences, NCCS Pune, India. The cell lines were grown in 25 cm^2 tissue culture flasks containing Minimum essential media (MEM media) with 10% fetal Bovine Serum (FBS), 1% L- glutamine and 50 μg/ml gentamycin sulphate at 37°C in CO_2 incubator in an atmosphere of humidified 5% CO_2 and 95% air. The cells were maintained by routine sub culturing in 25cm^2 tissue culture flasks twice a week. Ehrlich ascites carcinoma cells (EAC) and Dalton's ascites lymphoma cells (DLA) were obtained by Amala Cancer Research Center, Thrissur, Kerala, India and were maintained by weekly intraperitonial (i.p) inoculation of 10^6 cells/mouse in the laboratory. Both the cell lines maintained in the peritoneal cavity of Swiss albino mice were collected from an animal having 7 days old ascitic tumour by aspirating the ascetic fluid in sterile isotonic saline. The viable EAC/DLA cells were counted (Trypan blue indicator) under microscope. A fixed number of viable cells 10^6 cells were inoculated into the peritoneal cavity of each recipient mouse.

e) Animals

The experiments were done on 8-10 weeks old Swiss albino mice of either sex weighing 25-35 gm were procured from a registered breeder in Viveswarapura Institute of Pharmaceutical Sciences. Animals were maintained under controlled conditions of temperature (25 ± 3°C) and humidity (50 ± 5 %) and were caged in sterile polypropylene cages containing sterile paddy husk. The study protocol was authorized by Institutional Animal Ethics Committee (IAEC), Visveswarapura Institute of Pharmaceutical Sciences, Bangalore. (Registration No: 152/1999, renewed in 2012).

f) Cytotoxic screening

i. *Sulforhodamine B colorimetric assay for cytotoxic screening*

The monolayer cell culture of HEp-2 (Human epithelial carcinoma) was trypsinized and the cell count adjusted to 1.0 x 10^5 cell/ml using medium (MEM) supplemented with 10% FBS. To each well of the 96 well microplate, 1x10^4 cells in a volume of 0.1ml was added and incubated for 24 h in CO_2 incubator for cell adherence. After 24 h, cells were treated with combination of honey bee venom (5.7 µg/ml) and ethanol extract of *Zingiber officinale Roscoe* (100 µg/ml) in a volume of 100 µl. The plates were then incubated at 37°C for 3 days in 5% CO_2 atmosphere, and microscopic examination was carried out and observations recorded every 24 h. After 72 h incubation, cell monolayers were fixed with 10% (w/v) trichloroacetic acid and stained for 30 min, after which the excess dye was removed by washing repeatedly with 1% (v/v) acetic acid. The air-dried plates were stained with 100 µl of 0.4% Sulforhodamine B solution (SRB) (0.4 g of SRB was dissolved in 100ml of 10 mM Tris base solution) for 30 min. The unbound dye was then removed by rapidly washing four times with 1% acetic acid. The plates were shaken vigorously for 5 min. The absorbance was measured using micro plate reader at a wavelength of 510 nm (Orellana and Kasinski, 2016).

The percentage growth inhibition was calculated using the formula below:

$$\% \text{ Growth inhibition} = 100 \times (T - T_0) / (C - T_0)$$

T is the OD after exposure to certain concentration of drugs, T_0 is the OD at the start of drug exposure and C is the OD of untreated group which served as control.

ii. *Trypan blue dye exclusion method*

The combination of honey bee venom (20 µg) and ethanol extract of *Zingiber officinale Roscoe* (500 µg) was used for the preparation of the stock solution (520 µg/ml) in Phosphate buffered saline. Serial dilutions (25µg/ml, 50µg/ml, 75µg/ml, 100µg/ml of sample solution) were prepared in PBS. The amount of 200µl of sample solutions were poured in tubes and made up to 800 µl with PBS (Phosphate buffered saline). 100µl of EAC with a concentration of 10^6 cells/ml of Phosphate buffered Saline was added to the tubes. Solvent alone was served as control. 100 µl of trypan blue was added to all test tubes after 3 hours incubation. Ascetic tumour cells were counted by Cell Counting machine (Cedex,

Roche). The percentage of cytotoxicity (% dead cells) was calculated using the formula (Saluja et al., 2011): % Cytotoxicity= (Total cells counted- total viable cells) / Total cells counted×100

iii. *Brine shrimp lethality (BSL) bioassay*

The brine shrimp (Artemia salina) eggs were supplied from Brine Shrimp Direct, Ogaden, UT, USA. The tested chamber was partitioned into two equal parts with aeration supply. One part was lighted up with a bulb (60 W), while the other was darkened. Brine shrimp eggs were placed in the dark side and incubated at room temperature for 48h. The nauplii were moved towards the illuminated side after hatching, where they were collected by a Pasteur pipette. The tested sample were prepared by dissolving the combination of honey bee venom (0.26 mg) and ethanol extract of *Zingiber officinale Roscoe* (4.74 mg) in 5ml of DMSO to obtain 500 ppm stock solution and then diluted with sea water to get the requisite concentration (20, 50, 75, 100, 200 and 300 μg/ml). 5- Flourouracil (5-FU) was used as standard (10, 25, 50 and 75 μg/ml). Ten shrimps were transferred in each vial and made up of volume to 5ml with sea water. A drop of dry yeast suspension (3mg in 5ml sea water) was poured to each vial as food. Control vials were provided by adding equal volumes of distilled water. The vials were sustained under illumination. Survivors were counted by using 3 × magnifying glasses after 24h and the percentage of deaths (% Mortality) and IC_{50} value were calculated by using Finney Computer program (Meyer et al., 1982).

iv. *MTT assay*

Cells were subcultured in 96-well plates at a density of 10^3 cells per well with combination of honey bee venom (5.7 μg/ml) and ethanol extract of *Zingiber officinale Roscoe* extract (100 μg/ml) and cisplatin as standard (2.5 μg/ml) for 48 h in a final volume of 100 μl of media. Then, the medium was removed and 10 μL of MTT (5 mg/mL in PBS) was added to the fresh medium. After 4 h incubation at 37 °C, 100 μL DMSO was added to each well and plates were agitated for 1 min. The optical density (OD) was read using a conventional ELISA plate reader at 570nm. The percentage of viability was calculated as the following formula (Lai et al., 2012): (Viable cells)%=(OD of drug-treated sample/OD of untreated sample)×100

g) Treatment designed

For EAC/ DLA evaluation, Healthy, adult Swiss albino mice were divided into 6 groups consisting of 6 animals in each group. In EAC study, all the animals in each group, excluding group 1 received 10^6 EAC cells/mouse i.p. Group 1 considered as normal and group 2 was EAC control. Group 3 was administrated by standard drug cisplatin 3.5 mg/kg b.w, i.p, group 4, 5 and 6 were administered, orally with formulation of F_1 (honey bee venom (10 mg/kg) and ethanol extract of *Zingiber officinale Roscoe* extract (100 mg/kg), F_2 (honey bee venom (25 mg/kg) and ethanol extract of *Zingiber officinale Roscoe* extract (150 mg/kg) and F_3 (honey bee venom (50 mg/kg) and ethanol extract of *Zingiber officinale Roscoe* extract (200 mg/kg), respectively for 10 consecutive days. For DLA investigation, Group 1 served as normal control, group 2 was DLA control. Group 3 received standard drug cisplatin 3.5 mg/kg b.w, i.p, group 4, 5 and 6 were administered, orally with formulation of F_1, F_2 and F_3 respectively for 10 days.

h) Determination of survival time

At termination surviving animals of EAC/DLA tumour bearing mice were counted and the Mean survival time (MST) and the % increase in life span (% ILS) were calculated by the formula (Durairaj et al., 2009).

$$\% \text{ ILS} = \left(\frac{\text{MST of treated group}}{\text{MST of the control group}} - 1\right) \times 100$$

i) Body Weight Analysis

All mice were weighed on the day of tumour inoculation and the weekly intervals. Average gain in body weight and % increase in body weight was calculated by the formula 1 for EAC tumour bearing mice by and % reduction in body weight was calculated by formula 2 for DLA tumour bearing mice (Durairaj et al., 2009).

Formula 1: [% increase in body weight = (animal bw on resp. day/animal bw on day 0)-1 x 100]

Formula 2: [% reduction in body weight= (Gain in bw of control-gain in bw in treated gp/ gain in bw of control) x 100]

j) Hematological parameters

At the end of evaluation, on day 11, mice were anaesthetized by is oflurane. blood was collected from retro-orbital of mice for reckoning of white blood cell (WBC) count, red blood cells (RBC) count and the hemoglobin (Hb) content by standard procedures (Jain, 2005).

k) Statistical analysis

The data were manifested as mean ± S.E.M. The results were statistically analyzed by means analysis of variance (ANOVA) followed by Dunnett's post hoc test where the difference was contemplated significant if $p < 0.05$.

III. Results

a) In vitro cytotoxic screening

In SRB assay, combination of honey bee venom (5.7 μg/ml) and ethanol extract of *Zingiber officinale Roscoe* (100 μg/ml) displayed good cell growth inhibition with IC_{50} value of 53 μg/ml. In trypan blue dye exclusion assay, the mentioned formulation caused mortalities effectively with IC_{50} value of 37.49 μg/ml. In

BSL assay, the combination of honey bee venom (0.26 mg) and ethanol extract of *Zingiber officinale Roscoe* (4.74 mg) exhibited mortality of cells with IC_{50} value of 48.31 μg/ml. The combination of honey bee venom (5.7 μg/ml) and ethanol extract of *Zingiber officinale Roscoe* extract (100 μg/ml) was examined on normal Chinese hamster lung fibroblast cells (V79) and human breast adenocarcinoma cells (MCF-7) by MTT assay to assess its selectivity towards normal and cancer cells. On normal cells, V79, the IC_{50} of formulation and cisplatin as standard was found to be 89.61 μg/ml and 6.43 μg/ml respectively with 77% of cell survival for formulation. On MCF-7, the IC_{50} of formulation and cisplatin as standard was found to be 66.52 μg/ml and 1.91 μg/ml respectively with 61% of cell survival for formulation. So it means the combination of honey bee venom (5.7 μg/ml) and ethanol extract of *Zingiber officinale Roscoe* extract (100μg/ml) exhibited more cytotoxic activity towards cancer cells.

b) In vivo anticancer study

i. *Effect of combination of honey bee venom and ethanol extract of Zingiber officinale Roscoe on body weight in DLA/ EAC tumour bearing mice*

Figure 1 exhibits the percentage reduction in the body weight after treatment with of combination of honey bee venom and ethanol extract of *Zingiber officinale Roscoe* on body weight in DLA tumour bearing mice. The percentage reduction in body weight of DLA tumour bearing mice was found to be 65.38 % in the group treated with cisplatin. Maximum percentage reduction in body weight was found to be 55.21% in the group treated with F_3 formulation. Figure 2 displays the percentage increase in the body weight after treatment with of combination of honey bee venom and ethanol extract of *Zingiber officinale Roscoe* on body weight in EAC tumour bearing mice. Substantial rise in body weight was perceived in EAC control mice with a maximum gain (20.85 ± 0.41 %). Standard cisplatin and all herbal formulation treatment significantly declined the elevated body weight, hence the percentage increase in body weight was found to 3.68% and4.61% in group treated with cisplatin and F_3 formulation respectively.

ii. *Effect of combination of honey bee venom and ethanol extract of Zingiber officinale Roscoe on tumour volume in DLA/ EAC tumour bearing mice*

Figure 3 shows the effect of combination of honey bee venom and ethanol extract of *Zingiber officinale Roscoe* on tumour volume in DLA tumour bearing mice. The DLA inoculation increased the tumour volume (0.82 cm^3) in mice. The cisplatin treated group significantly ($a < 0.05$) exhibited maximum reduction in tumour volume (0.22 cm^3) in mice. F_3 formulation treated group has shown very effective in diminishing tumour volume to 0.29 cm^3 when compared with DLA control. Table 1 manifests the effect of combination of honey bee venom and ethanol extract of *Zingiber officinale Roscoe* on tumour volume in EAC tumour bearing mice. F_3 formulation treated group significantly ($r < 0.001$) decreased the tumour volume as compared to the EAC control group.

Table 1: The effect of combination of honey bee venom and ethanol extract of *Zingiber officinale Roscoe* on tumour volume in EAC tumour bearing mice

Treatment groups	Tumour volume (mL)
EAC	8.29± 0.16
Cisplatin	1.13 ± 0.31^{r}
F_1	3.77 ± 0.35^{r,z}
F_2	3.46 ± 0.46^{r,z}
F_3	3.15 ± 0.75^{r,z}

Values are mean ± S.E.M. (n=6). P values: $r < 0.001$, as compared with EAC control. $z < 0.001$, as compared to cisplatin as standard (by one way ANOVA followed by Dunnett's multiple comparison test)

iii. *Effect of combination of honey bee venom and ethanol extract of Zingiber officinale Roscoe on mean survival time and percentage increase in life span in DLA/ EAC tumour bearing mice*

Table 2 indicates the Effect of combination of honey bee venom and ethanol extract of *Zingiber officinale Roscoe* on mean survival time and percentage increase in life span in DLA tumour bearing mice. In DLA control group, the mean survival time was 20 days and it rose significantly up to 32 and 28 days with remedy by standard cisplatin group and F_3 formulation group respectively. The % ILS was observed to be 55.64% and 39.89% in DLA induced mice treated with standard cisplatin group and F_3 formulation group respectively. Table 3 revealed the Effect of combination of honey bee venom and ethanol extract of *Zingiber officinale Roscoe* on mean survival time and percentage increase in life span in DLA tumour bearing mice. The mean survival time in cisplatin treated mice found to be 25 days ($a< 0.05$). As compared to the EAC control group, F_3 formulation treated group has shown significant ($a < 0.05$) increase in the life span than the rest of the groups.

Table 2: Effect of combination of honey bee venom and ethanol extract of *Zingiber officinale Roscoe* on mean survival time and percentage increase in life span in DLA tumour bearing mice.

Treatment groups	MST (Days)	%ILS
DLA	20.63 ± 0.41	-
Cisplatin	32.11 ± 0.68[a]	55.64
F_1	23.17 ± 0.40 [b]	12.31
F_2	25.23 ± 0.45[a,b]	22.29
F_3	28.86 ± 0.70[a,b]	39.89

Values are mean ± S.E.M. (n=6). p values: a< 0.05, as compared with EAC control. b < 0.05, as compared to cisplatin as standard (by one way ANOVA followed by Dunnett's multiple comparison test)

Table 3: Effect of combination of honey bee venom and ethanol extract of *Zingiber officinale Roscoe* on mean survival time and percentage increase in life span in EAC tumour bearing mice.

Treatment groups	MST (Days)	%ILS
EAC	15.20 ± 0.51	-
Cisplatin	25.34 ± 0.38[a]	66.71
F_1	19.65 ± 0.71 [b]	29.27
F_2	22.93 ± 0.35[a,b]	50.85
F_3	23.77 ± 0.40[a,b]	56.38

Values are mean ± S.E.M. (n=6). P values: a< 0.05, as compared with EAC control, b < 0.05, as compared to cisplatin as standard (by one way ANOVA followed by Dunnett's multiple comparison test)

iv. *Effect of combination of honey bee venom and ethanol extract of Zingiber officinale Roscoe on hematological parameters in DLA/ EAC tumour bearing mice*

Table 4 presents the Effect of combination of honey bee venom and ethanol extract of *Zingiber officinale Roscoe* on hematological parameters in DLA tumour bearing mice. The total WBC count found significantly increased in DLA control group when compared with the normal group (c< 0.001). F_1, F_2 and F_3 formulations exhibited activity at per with cisplatin as standard and these differences were statistically non-significant for F_3 formulations treated group and y< 0.01, z< 0.001 for F_2 and F_1 formulations treated group respectively. RBC count and Hb count in DLA groups were significantly (c< 0.001) diminished as compared to normal group. Treatment with F_3 formulations revealed superior ascend in RBC count and Hb count when compared with DLA control group and restored these values towards normal. Treatment with the all three formulation significantly rose the RBC level and Hb content when compared to the EAC control [Figure 4,5]. F_3 formulation indicated better effect than other combinations as compared with EAC control (b < 0.05). The WBC count has been reduced significantly when compared with the EAC bearing mice and restored more towards the normal level with remedying by all three formulations. F_3 formulation displayed better activity when compared to the rest of the evaluated formulation and the cisplatin (c < 0.05) [Figure 6].

Table 4: Effect of combination of honey bee venom and ethanol extract of *Zingiber officinale Roscoe* on hematological parameters in DLA tumour bearing mice

Treatment groups	RBC count (x 10^9/mL)	WBC count(x10^4/mm^3)	Hb (g%)
NORMAL	4.81± 0.13	8.09± 0.10	14.78± 0.39
DLA	3.11± 0.6 [c]	19.78± 0.55 [c]	10.06± 0.42 [c]
Cisplatin	3.75±0.25 [r]	9.13± 0.23[r]	13.87± 0.45 [br]
F_1	3.27± 0.20[cq]	17.06± 0.52 [crz]	11.88± 0.17 [cx]
F_2	3.49± 0.52[cq]	15.58± 0.69 [bry]	12.53± 0.01 [c]
F_3	3.66± 0.30[cq]	11.52± 0.73 [r]	13.01± 4.10 [cq]

n = 6, Values are mean ±S.E.M, one way ANOVA followed by Dunnet's multiple comparison test. p values: b< 0.01, c< 0.001, compared to the normal group; q< 0.01, r<0.001, as compared with EAC control; x< 0.05, y< 0.01, z< 0.001, as compared cisplatin treated group.

IV. Discussion

The use of natural products to control or seize the carcinogenic activity issues an alternative to the use of typical allopathic remedy for therapy of the ailment (Balachandran and Govindarajan, 2005). Natural sources have been investigated in clinical researches and are being evaluated to comprehend their cancericidal properties against varied cancers (Balachandran and Govindarajan, 2005). Inevitably, this topical investigation was endeavoured to predict anticancer potential of combination of honey bee venom and ethanol extract of *Zingiber officinale Roscoe* against different cell lines. The cytotoxic screening of combination of honey bee venom and ethanol extract of *Zingiber officinale Roscoe* revealed the beneficial effect against cancer cell lines for *invitro* studies. Cytotoxic of anticancer drugs towards the normal cells are crucial problems in cancer remedy and engender the risk of promoting secondary malignancy (Shi et al., 2008). There has been a concerted research in the current years for the discovery of novel selective anticancer agents which show more cytotoxic activity towards cancer cells than normal cells. Therefore, in the present investigation of the cytotoxicity of combination of honey bee venom and ethanol extract of *Zingiber officinale Roscoe* on cancer (MCF-7) and normal (V79) cells was determined to check their selectivity that indicated more cytotoxic activity towards cancer cells with 61% of cell survival for formulation. In ascites /solid tumour models, a considerable rise in body weight of the animals was perceived in EAC/DLA control mice due to progressive accumulation of ascites tumour cells and rapid solid tumour growth respectively. The trustworthy criterion for determining the potential of any anticancer drugs is the extension of life span of animal (Dai and Mumper, 2010). The present study showed F_3 combination of honey bee venom and ethanol extract of *Zingiber officinale Roscoe* significantly ascended the life span in both EAC/DLA models. Additionally, the diminished volume of tumor and extended survival time of mice recommend the retarding effect of formula on cell division (Ames et al., 1993). Pivotal erythrocytopenia and anemia in cancer patients is mostly due to myelosuppression during chemotherapy (Mondal et al., 2014). Results acquired from investigation evinced F_3 combination of honey bee venom and ethanol extract of *Zingiber officinale Roscoe* reestablished the hematological parameters so it can alleviate erythrocytopenia and anemia in cancer patients. The prior phytochemical evaluation of honey bee venom has disclosed the presence of melittin which has antitumour activity (Orsolic et al., 2009). It is also one of the most potent inhibitors of calmodulin activity and a potent inhibitor of cell proliferation and clonogenicity. Calmodulin is vital for numerous processes that are crucial for normal cellular function, including the assembly and disassembly of microtubules, calcium extrusion from cells by a calcium-magnesium, ATPase, and the activation of many intracellular enzymes, such as phosphatases, protein kinases and cyclic nucleotide phosphodiesterase. Through interfering with any of these known functions, calmodulin inhibitors would be potentially toxic to cells. The evidence advocates that calmodulin inhibitors are cytotoxic to malignant cells (in both *invitro* and *invivo* investigation) which can postulated by either of following mechanisms: 1- Interfering with cell cycle by and block the movement of chromosome during metaphase that leads to inhibit the DNA synthesis, 2- Apoptosis and lysis of tumour cells (Orsolic et al., 2009). The previous phytochemical analysis has divulged the presence of [6] gingerol as the non-volatile pungent compounds in *Zingiber officinale Roscoe* (Poltronieri et al., 2014). [6] gingerol demonstrated antioxidant activity by modifying the redox status through inhibition of RNS (particularly peroxynitrite) (Radhakrishnan et al., 2014) and also inhibited the COX-2 expression by reason of blocking of P38 MAP kinase and NF- Kappa B (NF_kB) signalling pathway (Kim et al., 2005). There is ample evidence that COX-2 is overexpressed in about 85% of cancers. COX-2 is prostaglandin-endoperoxide synthase enzyme which catalyses the conversion of arachidonic acid to prostaglandin such as PGE2. PGE2 cause to increase the level of VEGF (Vascular endothelial growth factor) which lead to angiogenesis of cancer cells. It increases the level of AKt which enhances transcription factor of anti apoptosis in nucleus, additionally, it ascends the level of Bcl-2 which is an anti-apoptotic agent and hence it prevents the apoptosis process in cancer cells which causes to mortality of them. It means COX-2 inhibitor can induce apoptosis as well as anti-angiogenesis (Sharma and Sharma, 2007). Zingerone is another the non-volatile pungent compounds with phenolic nature in *Zingiber officinale Roscoe* (Poltronieri et al., 2014) .The data obtained from literature revealed that zingerone also has antioxidant activity against peroxynitrite and superoxide anion (Radhakrishnan et al., 2014).

V. Conclusion

Ergo, based on the current investigation, pharmacological potential of honey bee venom and ethanol extract of *Zingiber officinale Roscoe* towards anticancer activity was certified. Future evaluations can assess whether the combination of honey bee venom and ethanol extract of *Zingiber officinale Roscoe* may be more beneficial to impede the metastatic cancer or for remedy of established cancer.

VI. Acknowledgement

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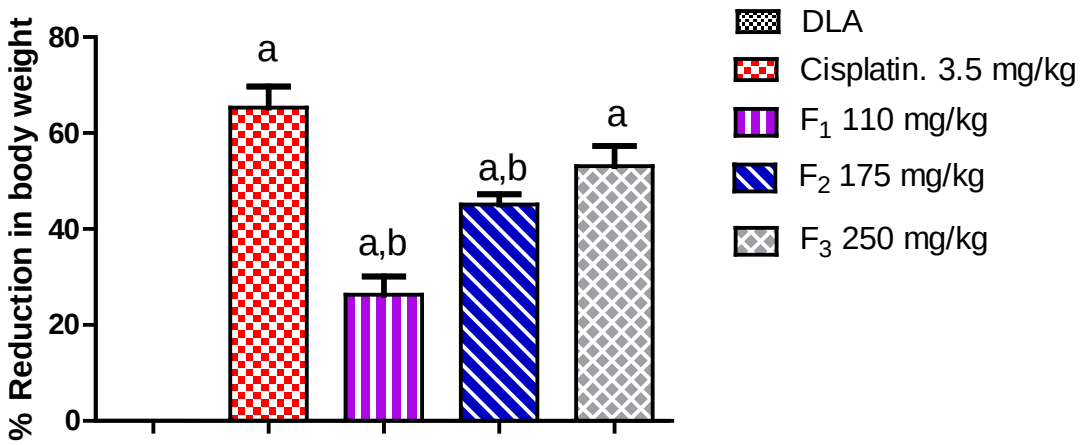

Figure 1: Effect of combination of honey bee venom and ethanol extract of *Zingiber officinale Roscoe* on body weight in DLA tumour bearing mice

n=6, values are mean ± SEM. Data were analysed by one way ANOVA followed by post hoc test. Where P values; a < 0.05 as compared to DLA control, b < 0.05 as compared to standard.

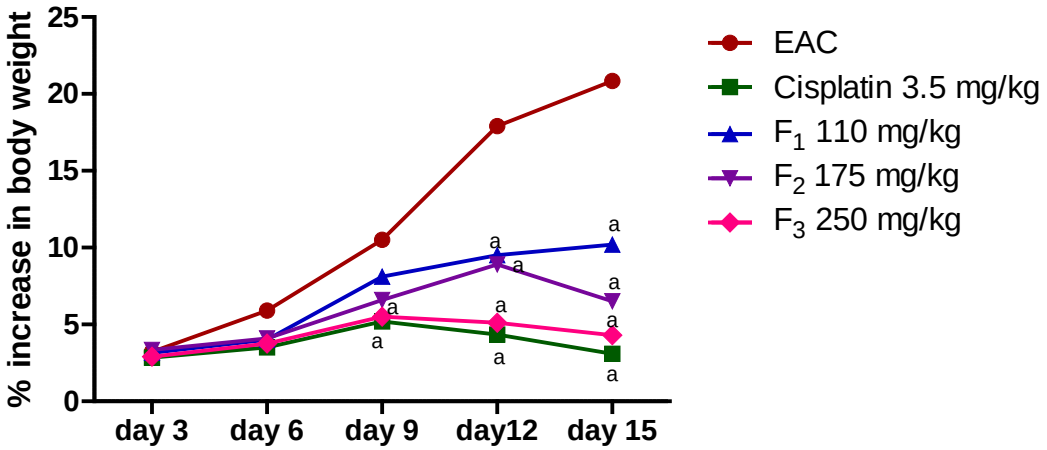

Figure 2: Effect of combination of honey bee venom and ethanol extract of *Zingiber officinale Roscoe* on body weight in EAC tumour bearing mice

n=6, values are mean ± SEM. Data were analysed by one way ANOVA followed by post hoc test. Where P values; a< 0.05 as compared to EAC control.

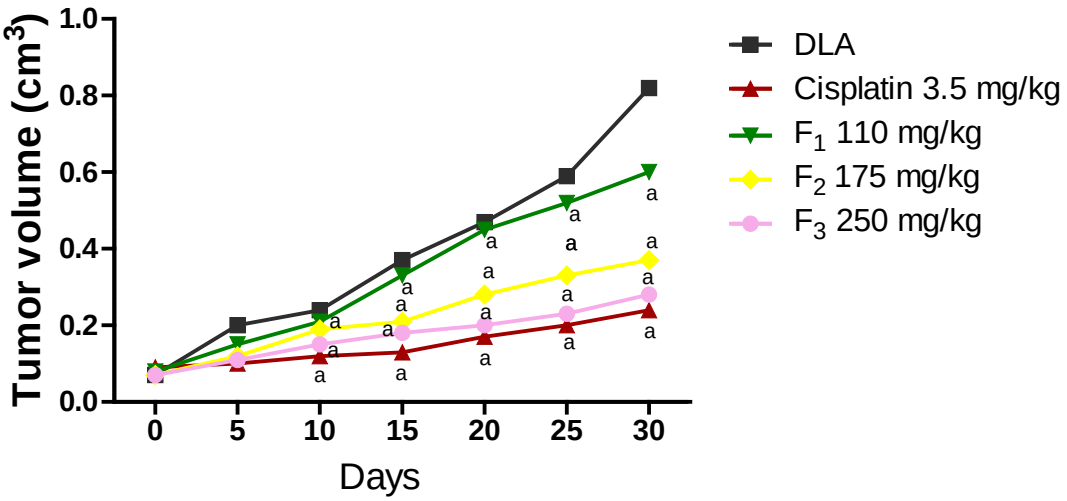

Figure 3: Effect of combination of honey bee venom and ethanol extract of *Zingiber officinale Roscoe* on tumor volume in DLA tumour bearing mice

n=6, values are mean ± SEM. Data were analysed by one way ANOVA followed by post hoc test. Where P values; a < 0.05 as compared to DLA control.

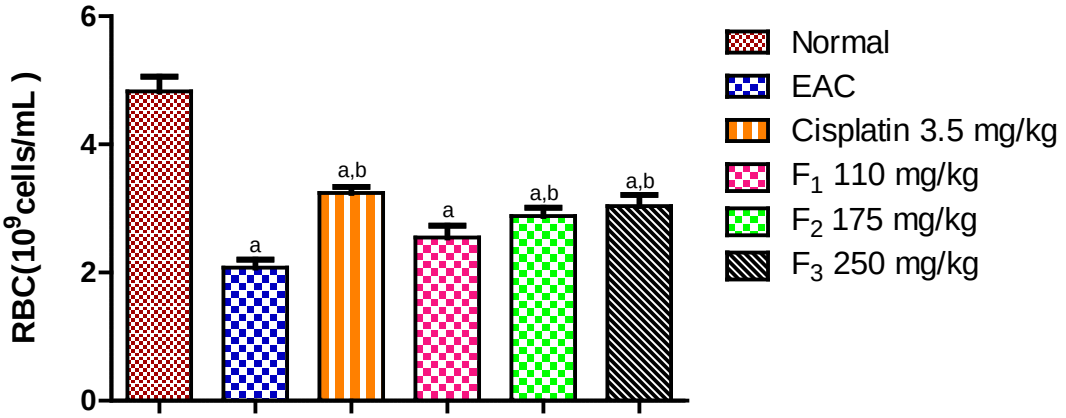

Figure 4: Effect of combination of honey bee venom and ethanol extract of *Zingiber officinale Roscoe* on RBC in EAC tumour bearing mice

n=6, values are mean ± SEM. Data were analysed by one way ANOVA followed by post hoc test. Where P values; a< 0.05 as compared to normal and b < 0.05 as compared to EAC control.

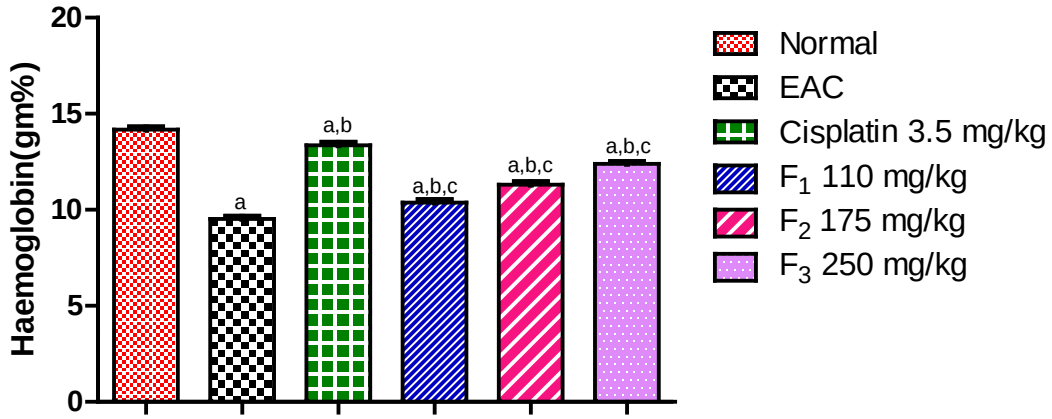

Figure 5: Effect of combination of honey bee venom and ethanol extract of *Zingiber officinale Roscoe*on Hb count in EAC tumour bearing mice

n=6, values are mean ± SEM. Data were analysed by one way ANOVA followed by post hoc test. Where P values; a< 0.05 as compared to normal, b < 0.05 as compared to EAC control and c< 0.05 as compared to cisplatin.

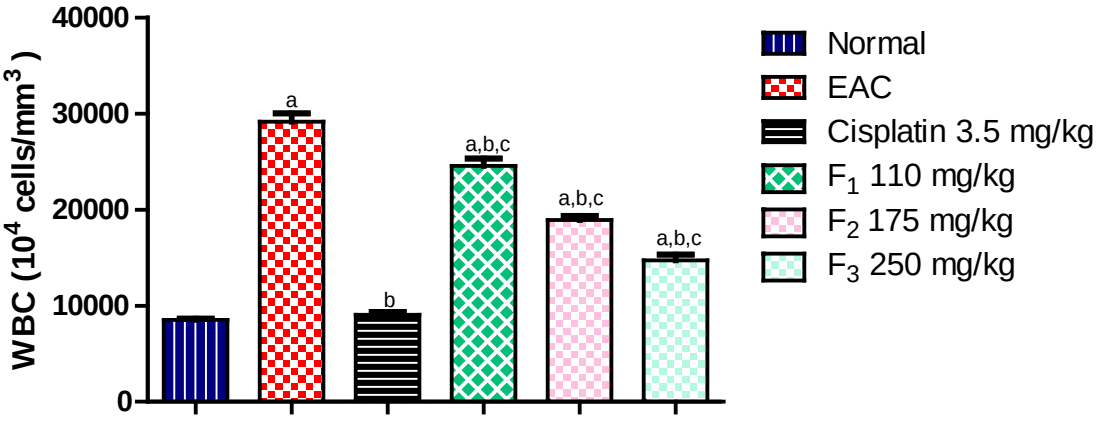

Figure 6: Effect of combination of honey bee venom and ethanol extract of *Zingiber officinale Roscoe* on WBC count in EAC tumour bearing mice

n=6, values are mean ± SEM. Data were analysed by one way ANOVA followed by post hoc test. Where P values; a< 0.05 as compared to normal, b < 0.05 as compared to EAC control and c< 0.05 as compared to cisplatin.

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Phytochemical, Antioxidant, Anti-Arthritic, Anti-Inflammatory and Bactericidal Potentials of the Leaf Extract of *Lactuca teraxacifolia*

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Abstract- *Lactuca taraxacifolia* is an important medicinal plant used locally in the treatment or prevention of many human diseases and illnesses. The aim of the study was to investigate the chemical composition, pH, TPC, TFC, TAA, carotenoid, antioxidant, anti-arthritic, anti-inflammatory and bactericidal activities. These were measured using GC-MS, pH meter, Folin-Ciocalteu's, AlCl3, 2,4-DNPH, acetone-hexane, DPPH, PTAC, BSA and agar-well diffusion methods respectively. The pH of the aqueous solution was 6.06. The GC and GC-MS analyses revealed the presence of 47 organic compounds making up 81.45% of the total percentage composition of the extract. The most abundant components were palmitic acid (8.5%), methyl-11-octadecenoate (7.7%), erythritol (7.5%), glycerol (6.5%), linolelaidic acid, methyl ester (6.2%) and phytol (5.5%). The TPC, TFC, TAA, ***β***-carotene, lycopene values were 3,041.50 ±0.00 µgmg^{-1} GAE, 59.05±0.00 µgmg^{-1} QE, 47.88±0.00 µgmg^{-1} AAE, 0.50 mgg^{-1} and 0.20 mgg^{-1}, respectively. The antioxidant IC50 and AAI values of the leaf extract were 0.75 µgml-1 and 53.33. The extract was capable of scavenging free radicals in a range of 72.07-90.91%. The PTAC value was 903.85±0.00 µgmg^{-1} AAE. The extract also gave high bovine anti-arthritic/anti-inflammatory values between 40-80% with IC_{50} value of 0.25 mgml^{-1}. The extract was active against all the tested bacteria with high zones of inhibition (11.0-30.0 mm). These results showed that the leaf extract of *L. taraxacifolia* could be used for the development of active drugs with broad activities for human being and animals.

Keywords: *lactuca taraxacifolia, phytochemical, pharmacological activities, antioxidant, anti-arthritic, anti-inflammatory, antimicrobial activities.*

GJMR-B Classification: *NLMC Code: QV 745*

Strictly as per the compliance and regulations of:

Phytochemical, Antioxidant, Anti-Arthritic, Anti-Inflammatory and Bactericidal Potentials of the Leaf Extract of *Lactuca teraxacifolia*

Ololade, Z.S. [α], Kuyooro, S.E. [σ], Ogunmola, O.O. [ρ] & Abiona, O.O. [ω]

Abstract- ***Lactuca taraxacifolia*** **is an important medicinal plant used locally in the treatment or prevention of many human diseases and illnesses. The aim of the study was to investigate the chemical composition, pH, TPC, TFC, TAA, carotenoid, antioxidant, anti-arthritic, anti-inflammatory and bactericidal activities. These were measured using GC-MS, pH meter, Folin-Ciocalteu's, $AlCl_3$, 2,4-DNPH, acetone-hexane, DPPH, PTAC, BSA and agar-well diffusion methods respectively. The pH of the aqueous solution was 6.06. The GC and GC-MS analyses revealed the presence of 47 organic compounds making up 81.45% of the total percentage composition of the extract. The most abundant components were palmitic acid (8.5%), methyl-11-octadecenoate (7.7%), erythritol (7.5%), glycerol (6.5%), linolelaidic acid, methyl ester (6.2%) and phytol (5.5%). The TPC, TFC, TAA, β-carotene, lycopene values were 3,041.50 ±0.00 μgmg^{-1} GAE, 59.05±0.00 μgmg^{-1} QE, 47.88±0.00 μgmg^{-1} AAE, 0.50 $mgg^{-1}$and 0.20 mgg^{-1}, respectively. The antioxidant IC_{50} and AAI values of the leaf extract were 0.75 μgml^{-1} and 53.33. The extract was capable of scavenging free radicals in a range of 72.07-90.91%. The PTAC value was 903.85±0.00 μgmg^{-1} AAE. The extract also gave high bovine anti-arthritic/anti-inflammatory values between 40-80% with IC_{50} value of 0.25 $mgml^{-1}$. The extract was active against all the tested bacteria with high zones of inhibition (11.0-30.0 mm). These results showed that the leaf extract of *L. taraxacifolia* could be used for the development of active drugs with broad activities for human being and animals.**

Keywords: lactuca taraxacifolia, phytochemical, pharmacological activities, antioxidant, anti-arthritic, anti-inflammatory, antimicrobial activities.

I. Introduction

Plants have limitless abilities to synthesize phytochemicals that have enormous therapeutic potentials (Suresh *et al*., 2012; Jain *et al*., 2015; Shittu and Akor, 2015). Secondary metabolites from plants are important component of alternative and complementary medicines as drugs derived from plants are still the main source of health care for the majority of rural dwellers (Shakya, 2016; Amira and Oloyede, 2017; Elamin, 2017). They are effective in the treatment of infectious diseases and simultaneously they also mitigate many of the side effects that are often associated with synthetic drugs (Rios and Recio, 2005; Jain *et al*., 2015).

Lactuca taraxacifolia (Willd) Schum. (*Asteraceae*) has been domesticated as a leafy vegetable in West Africa. *L. taraxacifolia* is used as a remedy for prevention and treatment of diseases such as measles, yaws, conjunctivitis, hyperthesion, cancer etc. It is reported to possess hypolipidaemic, antihypertensive effects (Adebisi, 2004; Obi *et al*., 2006; Sakpere and Aremu, 2008; Dairo *et al*., 2015). The leaves of *L. taraxacifolia* are used in stimulate lactation and also to induce multiple births in animals (Adinortey *et al*., 2012). The leaves are rubbed on limbs to aid walking in children. The milky latex of the plant is used to treat conjunctivitis (Sakpere and Aremu, 2008). This plant had been known for their nutritional quality for long; the plant is used as vegetable and eaten as salad or cooked as soups (Adinortey *et al*., 2012; Adetutu *et al*., 2013; Ruffina *et al*., 2016). It has been observed to be a good source of essential mineral elements (Soetan *et al*., 2010; Gbadamosi *et al*., 2012).

To the best of our knowledge, there is paucity of information on the chemical composition and pharmacological properties of *L. teraxacifolia* so far. Therefore, the present research was undertaken for with the aim at looking into the phytochemical, pH, ascorbic acid, total phenolic content, total flavonoid content, β-carotene, lycopene, antioxidant, anti-arthritic, anti-inflammatory and bactericidal potentials of the leaf extract of *L. taraxacifolia* from Nigeria.

II. Materials and Methods

a) Preparation of the extract

The leaves of the plant were collected from Benja village, Ota, Nigeria and it was authenticated as *Lactuca taraxacifolia* (Willd) Schum. (*Asteraceae*). Air dried and pulverised leaves were extracted with methanol. The mixture was then left in the dark at room temperatures for 3 days, and then subjected to filtration. The concentrated extract was refrigerated until used.

b) Measurement of pH

Pulverised leaves of *L. taraxacifolia* were soaked in distilled water for ~2.5 hr and then filtered. The pH

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values were measured in the fresh filtered solution using digital portable pH meter (Naka *et al*., 2016).

c) Gas Chromatography-Mass Spectroscopy Analysis

The leaf methanolic extract of *L. taraxacifolia* was analysed using Shimadzu GC-MS-QP2010 Plus (Japan). The separations were carried out using a Restek Rtx-5MS fused silica capillary column (5%-diphenyl-95%-dimethylpolysiloxane) of 30 m× 0.25 mm internal diameter (di) and 0.25 mm in film thickness. The conditions for analysis were set as follows; column oven temperature was programmed from 60-280 °C (temperature at 60 °C was held for 1.0 min, raised to 180 °C for 3 min and then finally to 280 °C held for 2 min); injection mode, Split ratio 41.6; injection temperature, 250 °C; flow control mode, linear velocity (36.2 cm/sec); purge flow 3.0 ml/min; pressure, 56.2 kPa; helium was the carrier gas with total flow rate 45.0 ml/min; column flow rate, 0.99 ml/min; ion source temperature, 200 °C; interface temperature, 250 °C; solvent cut time, 3.0 min; start time 3.5 min; end time, 24.0 min; start m/z, 50 and end m/z, 700. Detector was operated in EI ionization mode of 70 eV. Components were identified by matching their mass spectra with those of the spectrometer data base using the NIST computer data bank, as well as by comparison of the fragmentation pattern with those reported in the literature (Oyebanji and Ololade, 2017).

d) Determination of Total Phenolic Content (TPC)

The TPC of the leaf extract of *L. taraxacifolia* was determined using Folin-Ciocalteau method. 1000 μgml^{-1} of the extract was mixed with 1.0 ml of 10% Folin-Ciocalteu reagent in distilled water and then neutralized with 4 ml of 7.5% sodium carbonate solution. The sample was maintained at room temperature for 3 hrs with periodical mixing, the absorbance at 760 nm was measured using UV-visspectrophotometer. The index of TPC in the juice was determined as μgmg^{-1} of gallic acid equivalent (GAE) using an equation obtained from the calibration curve of gallic acid graph (Amira and Oloyede, 2017).

e) Total Flavonoid Concentration (TFC)

The TFC of the extract of *L. taraxacifolia* was determined by spectrophotometry, using aluminium chloride method and quercetin as standard. Briefly, 1.0 ml of the extract, 0.10 ml of 10% aluminium chloride ($AlCl_3.6H_2O$), 0.10 ml of sodium acetate ($NaC_2H_3O_2.3H_2O$) (1 M) and 2.80 ml of distilled water. After incubation for 40 min, absorbance was measured at 415 nm using a UV-Vis-spectrophotometer. To calculate the concentration of flavonoids, we prepared a calibration curve using quercetin as standard. The index of TFC concentration is expressed as quercetin equivalents (QE) in μg per mg of juice. All assays were carried out in triplicate (Formagio *et al*., 2014).

f) Determination of Total Ascorbic Acid (TAA)

0.1 ml (1000 μgm^{-1}) of the extract was added to 1.0 ml 2,4-dinitrophenylhydrazine (2,4-DNPH). It was allowed to stand for 30 min. and the absorbance was read in triplicate at 515 nm, using distilled water as blank. Ascorbic acid was used as a reference and for the calibration curve; result was expressed in microgram per milligram of ascorbic acid equivalent (Benites *et al*., 2015).

g) Determination of Carotenoid: Lycopene and β-Carotene Contents

200 mg of the leaves of *L. taraxacifolia* were homogenized with 10 ml of acetone-hexane mixture (ratio 4:6) to determine the lycopene and *β*-carotene contents. The homogenate was centrifuged at 5000 x g for 10 min at 4°C. Automatically, two phases separated and an aliquot was taken from the upper solution (supernatant) for measurement of optical density at 663, 645, 505, and 453 nm in a UV-Vis-spectrophotometer. The assays were carried out in triplicates, the results were mean ± SD with acetone:hexane as blank. Lycopene and β-carotene contents were calculated according to the equations:

Lycopene $= -0.0458A_{663} + 0.204A_{645} + 0.372A_{505} - 0.0806A_{453}$;

β-Carotene $= 0.216A_{663} - 1.22A_{645} - 0.304A_{505} + 0.452A_{453}$. Lycopene and *β*-carotene were finally expressed as mgg^{-1} fw.

Where A= absorbance recorded at specific wavelengths (Wei *et al*., 2013).

h) Determination of Free Radical Scavenging and Antioxidant Activities

i. *In vitro DPPH Assay*

The antioxidant and free radical scavenging of the extract of *L. taraxacifolia* were measured by using 2,2′- diphenyl-1-picryl-hydrazyl. Briefly, the reaction mixture (2.0 ml) consists of 1.0 ml of DPPH in methanol (0.004%) and 1.0 ml of various concentrations of the extract. It was incubated for 30 min. in dark, and then the absorbance was measured at 517 nm. The control was prepared by DPPH and methanol in place of sample. In this assay, the positive control was ascorbic acid. The percentage of inhibition can be calculated using the formula:

$$I\% = [(A_{blank} - A_{ext})/A_{blank}] \times 100$$

Where: A_{blank} is the absorbance of blank solution and A_{ext} is the absorbance of the extract. The dose response curve was plotted and IC_{50} value for the extract and the standard were calculated (Ololade *et al*., 2016).

Antioxidant Activity Index: The antioxidant activity index (AAI) was calculated as:

$$AAI = [\text{DPPH initial concentration}]/[IC_{50}]$$

AAI was classified as weak, when AAI < 0.5, moderate, when AAI ranged between 0.5-1.0, strong, when AAI ranged between 1.0-2.0, and very strong, when AAI > 2.0 (Ololade and Olawore, 2017).

ii. *Phosphomolybdate Total Antioxidant Capacity (PTAC) Assay*

The PTAC of the extract of *L. taraxacifolia* was determined with phosphomolybdenum using ascorbic acid as the standard. An aliquot of 1.0 ml of the extract solution was combined with 1.0 ml of reagent (0.6 M sulphuric acid, 28 μM sodium phosphate and 4 μM ammonium molybdate). The tubes were capped and incubated in a boiling water bath at 95 °C for 90 min. after the samples had cooled to room temperature, the absorbance of the aqueous solution of each were measured at 695 nm in UV spectrophotometer. The blank solution contained 1.0 ml of reagent solution and the appropriate volume of the same solvent was used for the sample and it was incubated under the same conditions as the rest of the samples. The total antioxidant capacity was expressed as equivalents of ascorbic acid (Borokini *et al*., 2017).

i) In-vitro Anti-Arthritic and Anti-Inflammatory Activities of the Extract on Inhibition of Protein Denaturation (Bovine Serum Albumin Assay)

In vitro anti-arthritic/anti-inflammatory activity of the extract was evaluated against protein denaturation method using BSA. Test solution (0.5 ml) composed of 0.05ml of the extract at different concentrations (1000–100 μgml^{-1}) and 0.45 ml of BSA (5% aqueous solution). Test control solution (0.5 ml) consisted of 0.05 ml of distilled water and 0.45mL of BSA (5% aqueous solution). Product control solution consisted of 0.05ml of the extract at different concentrations (1000–100 μgml^{-1}) and 0.45 ml of distilled water. Standard solution (0.5 ml) consisted of 0.05ml aspirin (3000 μgml^{-1}) plus 0.45ml of BSA (5% aqueous solution). Solutions were incubated at specific temperature (37 °C) for 20 min. Solutions were cooled and 2.5 ml of phosphate buffer (pH 6.4) was added to all the solutions and temperature was increased progressively up to 70 °C for 5 min. Absorbance of the resultant solution was measured using UV visible spectrophotometer at 660 nm. The percentage inhibition of protein denaturation was determined using the following formula:

$$I\% = [(A_{ts} - A_{pc})/A_{tcs}] \times 100$$

Where: A_{ts} is the absorbance of test solution; A_{pc} is the absorbance of the product control and A_{tcs} is the absorbance of test solution. The dose-response curve was plotted and IC_{50} value for the extract was calculated (Alamgeer *et al*. 2017).

j) In vitro Bactericidal Potential

The antibacterial potentials of the extract were carried out using Agar-well diffusion method against Gram-positive bacteria: (*Enterococcus faecalis*, *Micrococcus varians*, *Streptococcus agalactiae* and *Staphylococcus aureus*), Gram-negative bacteria: (*Escherichia coli*, *Klebsiella pneumoniae*, *Proteus mirabilis*, *Pseudomonas aeruginosa*, *Serratia marcescens* and *Salmonella typhimurium*). Bacteria were incubated and grown overnight at 37°C in Nutrient agar. The cultured bacteria were adjusted to 0.5 McFarland standards, 20 ml of sterilized Nutrient agar medium was homogenized and aseptically poured into sterile Petri dishes and plates were swabbed with inocula of the test organisms, and kept for 30 min. for adsorption. A sterile cork borer of 6mm in diameter was used to make uniform wells into which were added different concentrations (1000, 500 and 250 μgml^{-1}) of the extract. The plates were allowed to stay in a refrigerator for 1 hour to allow proper diffusion of the juice solution into the medium. Synthetic antibiotic gentamicin (10µg/disc) was used as positive control. The plates were then incubated at 37 °C for 24 hr before visual assessment of the inhibition zones. The zone of inhibition was measured to the nearest size in millimetre (mm) using standard rule. The assay was carried out in aseptic conditions in order to achieve consistency (Ololade *et al*., 2017).

III. Results and Discussion

a) pH of the Leaves of L. taraxacifolia

The pH of the distilled water leaf extract of *L. taraxacifolia* was 6.06 and within the standard limit (pH 3.40–6.10) that insures freshness for consumption (El-Sohaimy *et al*., 2015), this showed that the leaf of the plant had weak acidic property.

b) Chemical Constituent of the Leaf Extract of L. taraxacifolia

A total of 47 compounds were identified in the leaf methanolic extract of *L. taraxacifolia*, accounting for 81.45% of the total extract (Table 1), and the main constituents identified were palmitic acid (8.5%), methyl-11-octadecenoate (7.7%), erythritol (7.5%), glycerol (6.5%), linolelaidic acid, methyl ester (6.2%) and phytol (5.5%). The chemical composition of leaf extract of *L. taraxacifolia* investigated in this study was entirely different from what was obtained from other species of *Lactuca*. Previous studies on the chemical composition of fresh and dry leaves essential oils of *Lactuca sativa* from Sultanate of Oman showed that the composition was dominated by durenol (52.00% and 49.79%), thymol (11.55% and 10.73%) and α-pinene (5.11% and 4.05%) (Al-Nomaani *et al*., 2013). Likewise, *E*-Ethyl-(Z)-3-(4-acetylphenylthio) cinnamate (33.01%), acetate, (3α)-lup-20 (29)-en-3-ol (15.11%), 5,12-dihydroxy-, (5a,12α)-ergost-25-ene-3,6-dione (10.46%) and 3-ethoxy-1-(3H)-isobenzofuranone, (7.79%) were the most abundant component in GC-MS analysis of the methanolic entire extract of *Lactuca runcinata* (Kanthal *et al*., 2014).

Table 1: Chemical Composition of Leaf Methanolic Extract of *L. taraxacifolia*

Compound	Retention Index	Percentage Composition
valeric acid	811	0.5
glycerol	967	6.5
2,2'-oxybis[N,N-dimethylethanamine	991	0.2
β-(dimethylamino)ethylmethacrylate	1000	0.1
α-dodecene	1204	0.1
2-decenal	1212	0.3
propylhexedrine	1213	0.1
nonylcyclopropane	1216	0.05
erythritol	1229	7.5
10-undecenal	1239	0.5
n-decanol	1258	0.1
2-tridecene	1321	0.05
t-butylphenylcarbonate	1350	0.4
2-(dimethylaminoethyl)benzoate	1408	0.1
1-undecanethiol	1418	0.05
4-tetradecene	1421	0.05
pentitol	1491	1.5
N,N-dimethyldodecanamide	1504	3.0
tetradecyltrifluoroacetate	1613	0.1
selina-6-en-4-ol	1624	2.4
τ-muurolol	1640	1.4
S-[2-[N,N-Dimethylamino]ethyl]N,N-dimethylcarbamoylthiocarbohydroximate	1650	0.2
α-cadinol	1653	1.5
13-tetradece-11-yn-1-ol	1663	0.5
13-oxabicyclo[9.3.1]pentadecane	1690	0.5
trifluoroacetic acid, n-heptadecyl ester	1713	0.1
sorbitol	1752	0.5
1-octadecyne	1808	0.8
methyl-14-methylpentadecanoate	1814	4.5
cis,*cis*,*cis*-7,10,13-hexadecatrienal	1894	2.9
1-hexadecanethiol	1915	0.05
palmitic acid	1968	8.5
Phytol	2045	5.5
(Z,Z)- 9,12-octadecadien-1-ol	2069	0.5
methyl-n-octadecanoate	2077	2.5
methyl-11-octadecenoate	2085	7.7
linolelaidic acid, methyl ester	2093	6.2
globulol	2110	2.0
lineoleoylchloride	2139	0.5
9,9-dimethoxybicyclo[3.3]nona-2,4-dione	2148	3.5
farnesol	2350	2.5
5-benzoyl-N-(2-dimethylaminoethyl)furan-2-carboxamide	2388	0.1
2,2'-methylenebis[6-(1,1-dimethylethyl)-4-methylphenol	2398	1.3
pentadecanoic acid	2822	2.0
methylheptacosanoate	2972	0.4
lupeol	3270	0.3
γ-sitosterol	3351	1.4
Percentage Total		**81.45**

c) Total Phenolic Content (TPC)

The TPC of the extract was 3,041.50 μgmg^{-1} GAE (Table 2). This might be due to the presence of low molecular mass phenolic compound such as 2,2'-methylenebis[6-(1,1-dimethylethyl)-4-methylphenol in the leaf extract. TPC determined in this study for *L. taraxacifolia* was higher than those reported in other var. *longifolia* had the total phenolic contents of 235.31 mg CE/g extract (Edziri *et al*., 2011). The phenolic compound loses an H^+ ion to produce a phenolate ion, which reduces Folin-Ciocalteu reagent (Ahmed *et al*., 2015). Phenolic compounds are known as free radical terminators and strong chain breaking antioxidants, so this may contribute directly to

antioxidative action of the plant (Flora, 2009). Studies had shown that consumption of phenolic antioxidant prevents chronic disease such as cancer, cardiovascular diseases (CVD), diabetes, cirrhosis, malignancy, stroke and arthritis (Zhang *et al*., 2015; Działo *et al*., 2016). The outstanding pharmacological potential of phenolic compounds is due to their ability to block specific enzymes that cause inflammation. They also modify the prostaglandin pathways and thereby protect platelets from clumping (Okwu and Ezenagu, 2008, Okwu and Nnamdi, 2008; Osuntokun and Olajubu, 2014).

d) Total Flavonoid Content (TFC)

The TFC of the extract was 59.05 μgmg^{-1} QE (Table 2). Flavonoids limit the risk of degenerative diseases associated with oxidative damage. Flavonoids are very important plant secondary metabolites because their hydroxyl groups confer scavenging ability on them (Ghasemzadeh and Ghasemzadeh, 2011). The broad medicinal properties of flavonoids are attributed mainly to their antioxidant properties (Dai and Mumper, 2010; Sangeetha *et al*., 2016; Ganesan and Xu, 2017). Flavonoids slow down the oxidative degradation of lipids, improve the quality and nutritional value of food and biological response modifiers (Kumar, 2014; Mojzer *et al*., 2016). They have anti-inflammatory, antiallergenic, anti-viral, anti-aging, and anti-carcinogenic activities (Panche *et al*., 2016). Flavonoid exerts protection against chronic disease through the inhibition of cyclooxygenase and lipoxygenase activities in platelets and macrophages (Shukla *et al*., 2014; Karau *et al*., 2015; Fernandes *et al*., 2017).

e) Total Ascorbic Acid (TAA)

The TAA analysis of the investigated extract of *L. taraxacifolia* showed the presence of high amount ascorbic acid and its derivatives (Table 2). Ascorbic acid is a sugar acid lactone. It is synthesized in plants from glucose or other simple carbohydrates (Benites *et al*., 2015). Ascorbic acid is an essential micronutrient and antioxidant needed for normal metabolic function of the body. It plays an important role as a component of enzymes involved in the synthesis of collagens and carnitine. Ascorbic acid plays an important role in a number of metabolic functions including the activation of the B vitamin, folic acid, the conversion of cholesterol to bile acids and the conversion of the amino acid, tryptophan, to the neurotransmitter, serotonin (Naidu, 2003; Chambial *et al*., 2013).

Table 2: TPC, TFC and TAA of the Leaf Extract of *L. taraxacifolia*

TPC	TFC	TAA
3,041.50 ±0.00 μgmg^{-1} GAE	59.05±0.00 μgmg^{-1} QE	47.88±0.00 μgmg^{-1} AAE

Data are presented as the mean value ± S.D. of triplicate

f) Determination of Carotenoid: Lycopene and β-carotene

The carotenoid content (lycopene and *β*-carotene) of the extract was as shown in Table 3. Carotenoids are very potent natural antioxidants. Carotenoids are powerful antioxidants and are obtained primarily from fruit and vegetables. Different carotenoids, such lycopene and β-carotene have high potentials to decrease risk of disease. Carotenoids are important natural isoprenoid pigments synthesized in plants and have essential roles in protecting against excess light energy and oxidative damage. Their provitamin A activities and antioxidant properties were their most attractive functions. *β*-carotene is the major and most effective vitamin A precursor among carotenoids, and plays a crucial role in human health, protecting against age-related degenerative diseases, cardiovascular disease, certain cancers and vitamin A deficiency (Fiedor and Burda, 2014; Gul *et al*., 2015; Zeng *et al*., 2015; Burrows *et al*., 2017). Lycopene decreases vascular oxidative stress and inflammation. Lycopene shows high effects on the maintenance of NO levels, contributing to vasodilatation, even resulting in a more effective slowing of the progression of atherosclerosis, thereby reducing the cardiovascular risk (Mangge *et al*., 2014; Gammone *et al*., 2015; Assis *et al*., 2017). *β*-carotene is also important for the colour that it imparts to the food stuffs and as phytochemical for health benefits such as potent antioxidant and lowering the risk of heart diseases and certain types of cancers, enhancing the immune system and protection from age-related macular degeneration-the leading cause of irreversible blindness among adults (Eperjesi *et al*., 2015; Gul *et al*., 2015; Abdalla *et al*., 2016).

Table 3: *β*-carotene and Lycopene of the Leaf Extract of *L. taraxacifolia*

Carotenoid	Concentration (mgg^{-1})
β-carotene	0.50
Lycopene	0.20

g) Free Radical Scavenging and Antioxidant Activities

The percentage inhibitions of the extract at various concentrations (2000, 1000, 750, 500 and 100 μgml^{-1}) were 90.91, 90.22, 86.03, 84.64 and 72.07%, respectively. The methanolic leaf extract of *L. taraxacifolia* IC_{50} valueof 0.75 μgml^{-1} was twelve-fold lower than that of the reference compound ascorbic acid, which had an IC_{50} value of 9.0 μgml^{-1} and the AAI

of the extract was 53.33 (Table 4), while the related species such as *L. indica* with IC_{50} 12.2 μgml^{-1} for hot water extract (Wang *et al*., 2003) and leaf methanolic and aqueous extracts of *L. sativa* var. *longifolia* reported to have the DPPH antioxidant activities with IC_{50} of 3.5 and 4.1 μgml^{-1} respectively. Therefore, the leaf extract of *L. taraxacifolia* investigated in this study had higher Antioxidantpotential than the reference compound and related species.

h) Phosphomolybdate Total Antioxidant Capacity (PTAC)

The PTAC of leaf extract of *L. taraxacifolia* was found to be moderately high as shown in Table 4. The phosphor-molybdenum method is quantitative since the PTAC is express as ascorbic acid equivalents. Natural products had become the target of a great number of studies in finding the sources of potentially safe, effective and cheap antioxidants because accumulation of free radicals causes pathological conditions (Lu *et al*., 2010). On the basis of the results obtained in the present study, it was concluded that methanolic extract of *L. taraxacifolia* exhibited potent free radical scavenging activities which might be helpful in preventing the progress of various oxidative stress mediated disorders (Anil and Suresh, 2011; Rangasamy and Namasivayam, 2014; Dose *et al*., 2016). This clearly showed that the leaf of the plant scavenges free radicals, ameliorating damage imposed by oxidative stress in different disease conditions and served as a potential source of natural antioxidant (Subhadradevi *et al*., 2010; Ekaluo *et al*., 2015).

Table 4: Antioxidant Properties of the Leaf Extract of *L. taraxacifolia*

Extract and Reference Drug	DPPH IC_{50} μgml^{-1}	AAI	PTAC μgmg^{-1} AAE
Extract	0.75	53.33	903.85±0.00

i) Anti-Arthritic and Anti-Inflammatory Potentials

Leaf methanolic extract of *L. taraxacifolia* at different concentrations showed considerably high (14-80%) anti-arthritic/anti-inflammatory potential with IC_{50} 0.25 $mgml^{-1}$ against the denaturation of bovine serum albumin, as compared to the synthetic drugs (aspirin) (Table 5). This result is similar to what was obtained from the in vivo anti-arthritic test on *Ulva lactuca* from Mediterranean Sea shores in Alexandria (Ahmed *et al*., 2017). Synthetic drugs for rheumatoid arthritis have certain shortcomings and side effects. Natural products are being preferred over conventional drugs nowadays due to their easy and continuous availability, better compatibility, cost effectiveness, less potential of toxicity and side effects, higher safety, and improved efficacy (Ekor, 2014; Alamgeer *et al*., 2017). Denaturation of proteins is the cause of inflammation, lipodystrophy, hyperlipidaemia, vasomotor rhinitis, rheumatoid arthritis, atherosclerosis, cardiovascular diseases, cancer, kidney stones and diabetes mellitus (Kumar *et al*., 2011; Prabhu *et al*., 2014; Traore *et al*., 2014). Phytochemicals from plants that can prevent denaturation of protein inhibition therefore, would be useful for the development of anti-arthritic, anti-inflammatory and analgesic drug (Garcia-Garcia *et al*., 2014; Trivedi *et al*., 2017). Therefore, this study showed that the leaf extract of *L. taraxacifolia* is capable of preventing and controlling the denaturation of protein and thereby it inhibited the denaturation of protein and its effect was compared with the standard drug. The mechanism of denaturation involves alteration in electrostatic, hydrogen, hydrophobic and disulphide bonding (Arya *et al*., 2014; Elisha *et al*., 2016; Sumathi and Anuradha, 2017).

Table 5: Bovine Serum Albumin Anti-Arthritic/Anti-Inflammatory Activity of the Leaf Extract of *L. taraxacifolia* and Reference Drug

Conc. μgml^{-1}	% Inhibition	IC_{50} $mgml^{-1}$	% Inhibition of Aspirin 3000 μgml^{-1}
1000	80		
500	40	0.25	40
250	40		
100	14		

j) Bactericidal Potentials

The antibacterial screening of the leaf extract of *L. taraxacifolia* gave wide range of zones of inhibition against the tested strains of bacteria. The zones of inhibition of the leaf extract of *L. taraxacifolia* (11.0–30.0 mm) extract showed high bactericidal activities from sensitive to ultra-sensitive as compared to synthetic antibiotic (gentamicin) (Table 6). In this study extract demonstrated antibacterial activities which may explain anonymous claim on the traditional uses of *L. taraxacifolia* for treatment of bacteria infections. The antibacterial properties of the extract investigated in this study were more active than the extract of other *Lactuca* species such as leaves aqueous and methanolic extract of *L. sativa* from Saudi Arabia which showed moderate inhibitions against *S. aureus*, *S. pyogenes*, *B. subtilis*, *E. coli* and *P. aeruginosa* between 9.0–14.0 mm (Bhat and Al-Daihan, 2014). Likewise, methanolic extract of *L.*

runcinata also showed inhibitions (8.4-17.8 mm) against *S. aureus*, *E. coli*, *S. typhi, S. paratyphi*, *P. mirabilis, P. aeruginosa and K. Pneumoniae* which are similar to the bactericidal activities of the leaf extract of the plant investigated in this study (Kanthal *et al*., 2013). Multi-drug resistance bacteria are major public health problems today, but secondary metabolites from plants have synergistic potential to tackle these problems, in that they possess antibiotic properties, safer than synthetic drugs, offering profound therapeutic benefits and more affordable treatment (Aiyegoro and Okoh, 2009; Okigbo *et al*., 2009; Auria *et al*., 2014; Toner *et al*., 2015; Dhillon *et al*., 2015; Igbinosa *et al*., 2016; Amuka *et al*., 2017).

Table 3: Zones of Inhibition (mm) Showing the Bactericidal Properties of the Leaf Extract of *L. taraxacifolia*

	Leaf Extract			Synthetic Antibiotic GEN
Conc. (μgml^{-1}) / Organisms	1000	500	250	10μg
E. coli (-)	13	11	11	12
E. faecalis (+)	18	18	18	12
K. pneumoniae (-)	30	30	30	20
M. varians (+)	24	20	18	18
P. aeruginosa (-)	11	11	11	20
P. mirabilis (-)	11	11	11	16
S. agalactiae (+)	12	12	12	-
S. aureus (+)	18	17	14	-
S. marcescens (-)	15	15	15	30
S. typhimurium (-)	11	11	11	18

Key note: Resistant (--), not sensitive (<8 mm), sensitive (9–14 mm), very sensitive (15–19 mm) and ultrasensitive (>20 mm)

IV. Conclusion

This study had demonstrated the medicinal properties of the methanolic leaf extract of *L. taraxacifolia* and showed that this therapeutic effect could be attributed to the active secondary metabolites such as phenolic and flavonoid compounds in the plant. Leaves of the plant contain wide range of health-promoting phytochemicals. This work also contributed to the appreciation of the nutritional and medicinal values of the plant. The characteristics of the leaf as a dietary source of antioxidant and antibiotic were also pointed out. The leaves of the plant possessed high antioxidant activity which might be helpful in preventing or slowing the progress of various oxidative stress related disorders and therefore can be used in food and pharmaceutical industries.

Conflict of Interest Statement: The authors declared that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Hepatoprotective Activity of *Aralia racemosa* L. and its Triterpenoid and Steroid Compounds against Paracetamol – Induced Liver Injury in Albino Wistar Rat

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Abstract- *Objective:* There exists a deficit of reliable hepatoprotective drugs in modern medicine to prevent and treat drug-induced liver damage. The root of *Aralia racemosa* L. belonging to family Araliaceae is pre-owned long established for their hepatoprotective effect. The prevailing research was accompained to identify and isolate the phyto-constituents of Aralia racemosa L. root methanolic extract (MEAR) for its hepatoprotective effect.

Materials and Methods: The dried root of *A. racemosa* was extracted with methanol and partitioned between Petroleum ether, chloroform, ethyl acetate, and n-butanol. The organic layer was fractionated by various stationary phases and identified by using spectral analysis. MEAR (200 and 400 mg/kg, p.o.) and isolated compounds were assessed for its hepatoprotective activity in PCM-induced liver toxicity in Rats. The hepatoprotective activity was assessed from biochemical and histopathological studies.

Keywords: *hepatoprotective; ursolic acid; oleanolic acid; silymarin; aralia racemosa L.; β-sitosterol.*

GJMR-B Classification: *NLMC Code: QV 738*

Strictly as per the compliance and regulations of:

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D S N B K Prasanth [α], A Srinivasa Rao [σ] & Y Rajendra Prasad [ρ]

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Results: Phytochemical investigation of the roots of Aralia racemosa L. (Araliaceae) afforded four known Phytoconstituents identified as Stigmasterol (1), ß-Sitosterol (2), Ursolic acid (3) and Oleanolic acid (4). The structures of those phytoconstituents have been elucidated based on spectral information analysis. Stigmasterol and Ursolic acid were isolated from this plant for the first time. The PCM intoxication leads to histological and biochemical deteriorations. The treatment with MEAR and the isolated compounds 1 to 4 significantly lowered the elevated levels of SGOT, SGPT, ALP, TB, as well as regressive the hepatic damage towards normal which further supports the hepatoprotective activity of MEAR.

Conclusion: This result strongly supports that MEAR had a significant protective effect against Paracetamol (PCM) - induced liver injury due to Phytosterols i.e., Stigmasterol, ß-Sitosterol and Triterpenes i.e., Oleanolic Acid and Ursolic acid.

Keywords: hepatoprotective; ursolic acid; oleanolic acid; silymarin; aralia racemosa L.; β-sitosterol.

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I. Introduction

The liver is an essential metabolic organ that will have secretory and excretory functions. It has a preeminent significance in the body because of its decisive functions like the removal of toxins associated with endogenous and exogenous substances like xenobiotics, viral infections, chronic alcoholism, bile secretion etc. liability to all the above complications results in liver failure. Worldwide the fatality and morbidity of liver disorders raise every year. Almost 20,000 fatalities and 2,50,000 new cases identified every year. Liver damage or failure is often related to hepatocytes necrosis and increased levels of biochemical parameters such as SGOT, SGPT, SALP and Total bilirubin levels. Even though remarkable improvements in modern medicine, there are hardly any reliable medicines which protect the liver from damage and/or assist in regeneration of hepatic cell. There are wide ranges of drugs and natural plants available in the commercial market for liver disorders. These products do not get rid of all of the liver conditions. For that reason, there is the urge to disclose the proper treatment (Nallamilli et al., 2013). Many plants that belong to family Araliaceae have been identified as hepatoprotective like *Panax vietnamensis* (Tran et al., 2001), *Acanthopanax koreanum* (Nan et al., 2008), *Dendropanax morbifera* (Bae et al., 2015), *Panax ginseng* (Kim et al., 2016) and *Schefflera kwangsiensis* (Wang et al., 2014).

Aralia racemosa L. (family: Araliaceae) is a plant which is native to the equatorial and fructiferous region of the world. The genus *Aralia* consists of 71 species of plants distributed all over Asia, Mexico, North America, and South America. In 1994 Smith identified the North American species of Araliaceae and recognized the following eight species of *Aralia i.e., A. racemosa, A. californica, A. nudicaulis, A. spinosa, A. hispida, A. humilis, A. regeliana* and *A. scopulorum.* Standley recognized five species of *Aralia* from Mexico: *A. scopulorum, A. regeliana, A. humilis, A. pubescens, and A. racemosa* (Wen, 2011). Traditionally, *A. racemosa* roots has a wide range of reputed medicinal

applications as carminative, antiseptic, in cough preparations, pain in the breast, mortifications, rheumatism, Whooping cough, skin diseases, pleurisy, diaphoretic, diuretic, pulmonary diseases, asthma, diarrhea, stimulant, expectorant, syphilis, inflammation and hay fever (Duke & Duke, 2006; Quattrocchi, 2012). Only a few pharmacological properties have been reported from this plant such as antioxidant, antidiabetic (McCune & Johns, 2002, 2003) and antitubercular (Grange & Davey, 1990). Few phytoconstituents are documented with this plant including triterpenoid saponins i.e., oleanolic acid, sterols i.e., β-sitosterol and Diterpenoids i.e., *ent*-Kaurenoic acid, continentalic acid (Clement et al., 2013; McCune & Johns, 2002). As the genus *Aralia* is enriched with triterpenoid saponins, which may possess hepatoprotective activity based on precedent studies (Bae et al., 2015; Kim et al., 2016; Nan et al., 2008; Tran et al., 2001; Wang et al., 2014).

Therefore, the present research was sketch out to identify and isolate the chemical compounds of *Aralia racemosa* L. (*A. racemosa*) root methanolic extract for its hepatoprotective effect in albino Wistar rats.

II. Materials and Methods

a) Plant material

Aralia racemosa L. root was procured in the month of September 2015, from Sri Venkateswara University, Andhra Pradesh. It was identified and authenticated by K. Madhavachetty, plant taxonomist, Department of Botany, Sri Venkateswara University, Tirupati, Andhra Pradesh and voucher specimen of the plant (No 1489) was deposited at the herbarium for future reference.

b) Chemicals, Materials, Instrumentation, and Drugs

All the chemicals used for this experiment were of analytical grade. Paracetamol (E. Merck), silymarin (Sigma Chemical Co.,) and thiobarbituric acid (Sigma Chemical Co.,). Silica gel for column chromatography (CC) was performed on silica gel (Merck silica gel 60H, particle size 5 - 40 μm). Thin layer chromatography (TLC) was performed on Merck aluminum-backed plates, pre-coated with silica (0.2 mm, 60F254). The ultraviolet (UV) spectra of the compounds in methanol were recorded on a Shimadzu UV-1800 spectrophotometer. The IR spectra were recorded using KBr discs on a NICOLET 380 FT-IR spectrometer (Thermo Fisher Scientific, France) in the range of 400 to 4000 nm. The mass spectrum in ESI mode was obtained using LCMS2010A (Shimadzu, Japan) having probes APCI & ESI. Nuclear magnetic resonance 1 H NMR and 13C NMR spectra were recorded at 400 MHz, respectively on a Bruker DRX-400 spectrometer (Bruker Bio spin Co., Karlsruhe, Germany) in DMSO and $CDCl_3$ with tetramethylsilane as an internal standard. Melting points were determined using Royal Scientific RSW 138A melting point apparatus. Further elemental analysis of the compounds was performed on a Perkin Elmer 2400 elemental analyzer. Diagnostic kits for the estimation of serum glutamicoxaloacetic transaminase (SGOT), serum glutamic pyruvic transaminase (SGPT), alkaline phosphatase (ALP), serum cholesterol and serum bilirubin were manufactured by Ranbaxy Diagnostics Ltd., New Delhi, India. The standard orogastric cannula utilized for oral drug administration.

c) Test animals

The study was carried out on Wistar albino rats (160-200g) of either sex (mahaveer Enterprises, Hyderabad.) and was kept at an animal house in V. V. Institute of Pharmaceutical Sciences, Gudlavalleru bearing CPCSEA registration number 1847/PO/Re/S/16/CPCSEA. They were allowed to take standard pellet food and water *ad libitum*. Before the experiment, the rats were kept in standard environmental conditions at room temperature 25-27°C relative humidity (55 ± 5)% and 12 h light/12 h dark cycle for 7 days. All rats received humane care in accordance with the "Guide for the Care and Use of Laboratory Animals" (Kiran, Raju, & Rao, 2012).

d) Preparation of Extracts and Solvent fractionation

The freshly collected roots were shade dried and pulverized. The powder (3 Kg) was treated with petroleum ether for the removal of fatty and waxy material. Then it was air dried and macerated with methanol, filtered and concentrated at 45°C in Buchi rotavapor. The weight of methanolic extract obtained was 73g (7.3% w/w yield). The methanolic extract had been suspended in distilled water in a separating funnel and partitioned sequentially with petroleum ether, chloroform, ethyl acetate and n-butanol to acquire fractions in these solvents. Eventually, left residual aqueous fraction at the end was collected. The solvents were removed on a rotary evaporator at low pressure to obtain dried fractions. These extracts were subjected to preliminary phytochemical screening and these extracts were stored in the refrigerator at 4°C for further use(Ahmed, Saeed, Shakeel, Fatima, & Arshad, 2015).

e) Isolation of Constituents

Petroleum ether extract (PEE) was subjected to silica-gel (100–200 mesh) column (length 100 cm and diameter 3 cm) chromatography (elution rate of 2 ml min−1 flow with a total elution of 200 ml) and eluted with Petroleum ether and ethyl acetate in different proportions. The consequent fractions (Fr) were collected and spotted over pre-coated silica gel F254 plates (20 × 20 cm, Merck, Germany). The optimum resolution was achieved in the hexane, ethyl acetate and formic acid (7.5: 2: 0.5 v/v) solvent system and the plates were sprayed with anisaldehyde–sulphuric acid reagent to visualize the spots. The fractions showing similar spots were pooled together and concentrated. The fractions which showed prominent spots were taken

up for spectral studies which result in the identification of 4 compounds. The compounds PC-1 and PC-2 were identified as phytosterols by Libermann–Burchard's test (Figure 1a). The chloroform fraction was subjected to chromatography on silica gel (60–120 mesh, Merck) eluted with ethyl acetate-hexane (7:3) solvent system. Repeated chromatography to give major two pentacyclic triterpenoids i.e., PC-3 and PC-4 (Figure 1b) (Hossain & Ismail, 2013; Vasconcelos et al., 2006).

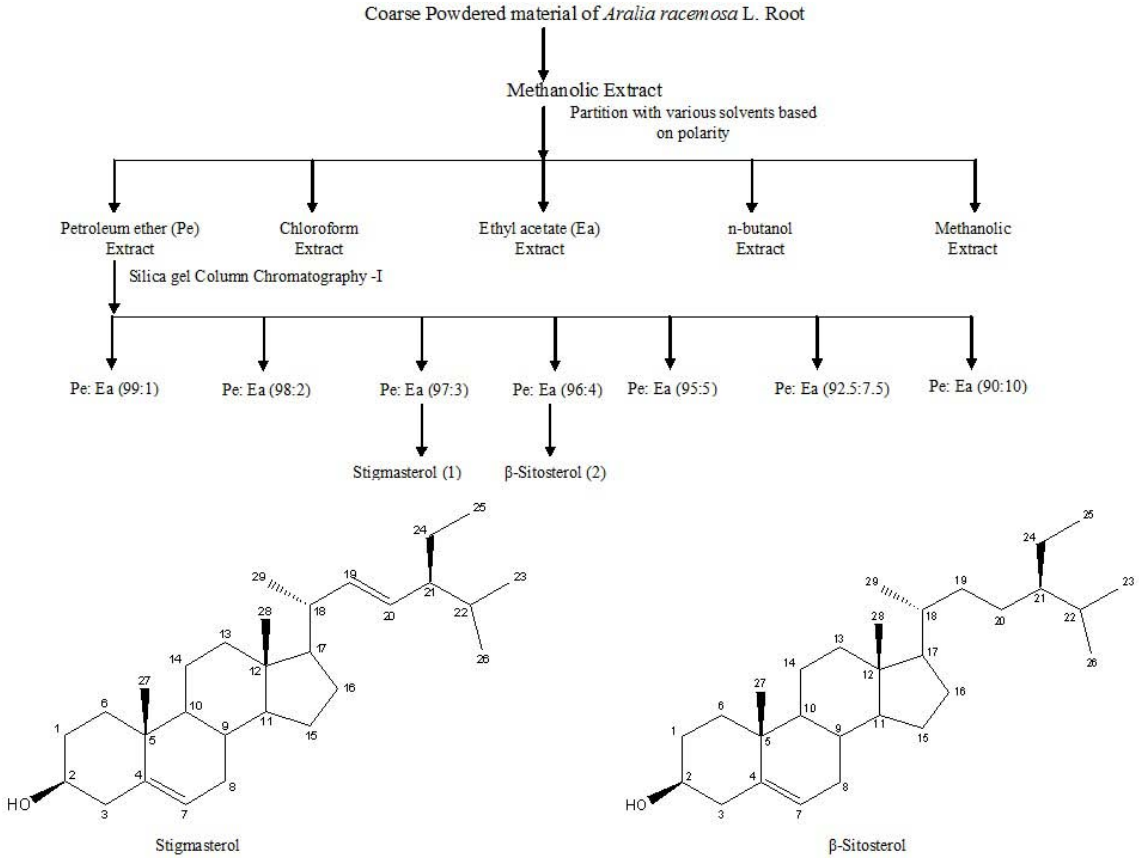

Fig. 1 (a): Separation scheme of active compounds from the Petroleum ether fraction of *Aralia racemosa* L. root and structures of isolated compounds.

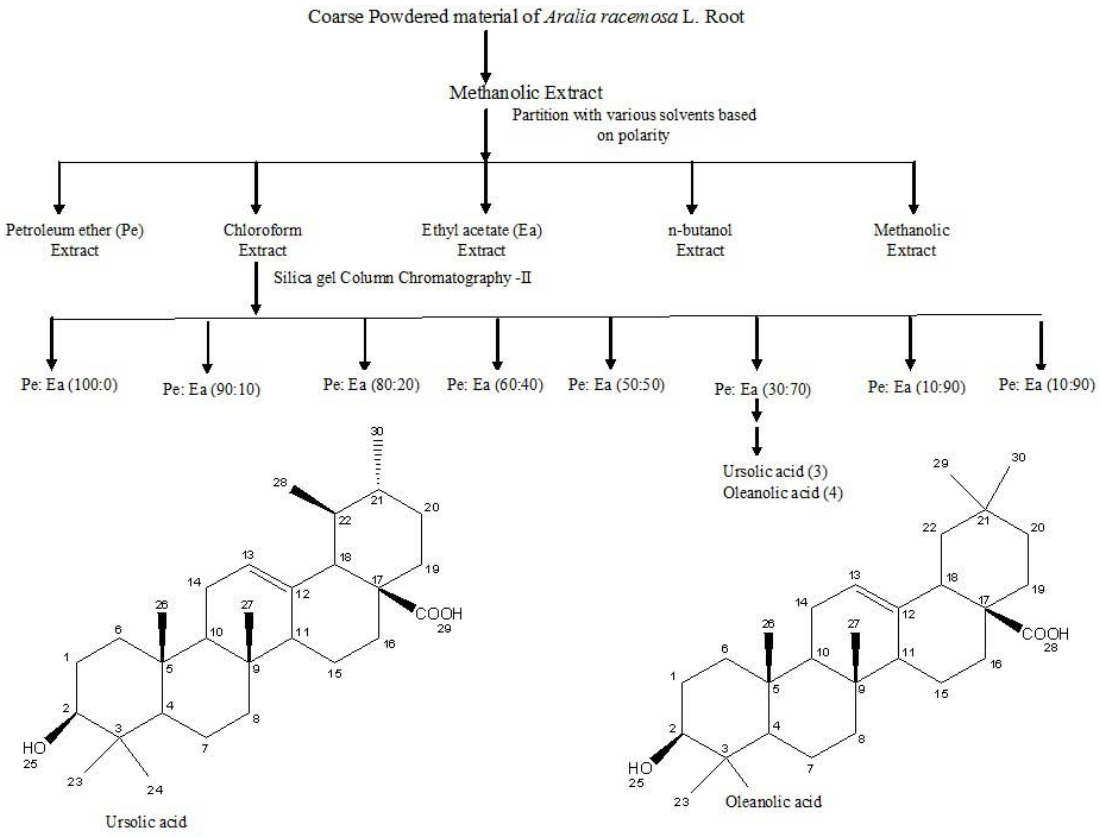

Fig. 1 (b): Separation scheme of active compounds from the chloroform fraction of *Aralia racemosa* L. root and structures of isolated compounds.

f) Phytochemical Screening

The methanolic extract of *A. racemosa* L. root was subjected to qualitative chemical analysis by using standard procedures as follows.

The phytochemical screening of carbohydrates was detected by molisch's test; Proteins was detected by using two tests namely Biuret test and millon's test and amino acids by Ninhyrdin's test; Steroids was identified by salkowski, Libermann- Burchards and Libermann's test; Alkaloids was identified with freshly prepared Dragendroff's Mayer's, Hager's and Wagner's reagents and observed for the presence of turbidity or precipitation. The flavonoids were detected using four tests namely Shinoda, sulfuric acid, aluminum chloride, lead acetate, and sodium hydroxides. Tannins were detected with four tests namely gelatin, lead acetate, potassium dichromate and ferric chloride. The froth, emulsion, and lead acetate tests were applied for the detection of saponins. The steroids were detected by (acetic anhydride with sulfuric acid) and (acetic chloride with sulfuric acid) tests. Sample extracted with chloroform was treated with sulfuric acid to test for the presence of terpenoids. Ammonia solution and ferric chloride solutions were used for the presence of anthraquionones (Alam & Najum us Saqib, 2015; Harborne, 1973; Khandelwal, 2008; Raaman, 2006; Singh, Khosa, Mishra, & Jha, 2015).

g) Acute toxicity study

To evaluate the toxicity of *A. racemosa* extract the acute toxicity study was performed based on OECD (Organization for Economic Cooperation and Development) 423 guidelines up to the dose of 2000 mg/Kg. The rats were observed for 1 h continuously and then hourly for 4 h and finally after every 24 h up to 14 days for any physical signs of toxicity, such as writhing, gasping, palpitation and decreased respiratory rate or mortality. No animals died. Therefore the LD50 is greater than 2000 mg/kg. Pre-screening investigation with 200 and 400mg per body weight was done (Kiran et al., 2012).

h) Evaluation of Hepatoprotective activity

i. *Induction of Paracetamol-induced hepatotoxicity*

Male Wistar rats weighing 150–180 g were used for the study. Animals were divided into 9 groups of 5 animals each. All rats were treated orally for 5 consecutive days (Kalantari, Forouzandeh, Azemi, Rashidi, & Goudarzi, 2013).

Group I: (normal control) received 0.5% tween 80 (1 ml/kg b.wt. p.o.) for 5 days.

Group II: (toxic group) received 0.5% tween80 (1 ml/kg b.wt. p.o.) for 5 days and PCM (2 g/kg b.wt. p.o) on the 5th day.

Group III: (test group), received the methanolic extract of *A. racemosa* (200 mg/kg b. wt. p.o) for 5 days and PCM (2g/kg) was administered 1 hour after the last administration of crude extract.

Group IV: (test group), received the methanolic extract of *A. racemosa* (400 mg/kg b. wt. p.o) for 5 days and PCM (2g/kg) was administered 1 hour after the last administration of crude extract.

Group V: (test group), received Oleanolic acid (20 mg/kg b. wt. p.o) for 5 days and PCM (2g/kg) was administered 1 hour after the last administration of crude extract.

Group VI: (test group), received Ursolic acid (20 mg/kg b. wt. p.o) for 5 days and PCM (2g/kg) was administered 1 hour after the last administration of crude extract.

Group VII: (test group), received β-Sitosterol (20 mg/kg b. wt. p.o) for 5 days and PCM (2g/kg) was administered 1 hour after the last administration of crude extract.

Group VIII: (test group), received Stigmasterol (20 mg/kg b. wt. p.o) for 5 days and PCM (2g/kg) was administered 1 hour after the last administration of crude extract.

Group IX: (Standard group), received Silymarin (25 mg/kg b. wt. p.o) for 5 days and PCM (2g/kg) was administered 1 hour after the last administration of crude extract.

ii. *Assessment of liver function test*

Animals had been sacrificed and blood was obtained directly via retro-orbital plexus. Serum was separated after coagulating at 37 °C for 30 min and centrifuged at 3000 rpm for 15–20 min. Serum was used for the estimation of biochemical parameters like serum glutamate pyruvate transaminase (AST), serum glutamate oxaloacetate transaminase (ALT) and alkaline phosphatase (ALP) and the liver tissues collected were subjected to histopathology. AST, ALT, and ALP were measured by using diagnostic kits (Kalantari et al., 2013).

iii. *Histopathological studies*

Livers of different groups were fixed in 10% buffered neutral formalin for 48 h and then with a bovine solution for 6 h. Paraffin sections were taken at 5 mm thickness, processed in alcohol-xylene series and were stained with alum hematoxylin and eosin. The sections were examined microscopically for histopathological changes (Kiran et al., 2012).

i) Statistical analysis

All values expressed as mean±SEM; n=5 rat in each group, by one-way ANOVA followed by Tukey's Multiple Comparison Test using Graph pad Prism-5 software. $p<0.05$ was considered as significant (Kiran et al., 2012).

III. Results

a) Acute Toxicity Studies

The methanolic extract of A. racemosa roots, when orally administered in the dose of 2000 mg/kg body wt. did not produce any significant changes in the autonomic or behavioral responses, including death during the observation period.

b) Phytochemical Screening

The phytochemical screening for various extracts viz., petroleum ether, chloroform, ethyl acetate, methanol, n-butanol, and water was carried out and results were displayed in Table 1.

Table 1: Preliminary phytochemical screening of various extracts of Aralia racemosa L. root

Phytoconstituents	Method	Pet. ether Extract	Chloroform Extract	Ethylacetate Extract	Methanolic Extract	n-butanol Extract	Aqueous Extract
Flavonoids	Shinoda Test	-	-	+	+	-	+
	Zn+HCl test	-	-	+	+	-	+
	Lead acetate Test	-	-	+	+	-	+
Volatile oil	Stain test	+	-	-	+	-	+
Alkaloids	Wagner Test	-	+	-	+	-	+
	Hager's Test	-	+	-	+	-	+
Tannins & Phenols	$FeCl_3$ Test	-	-	-	+	+	+
	Potassium dichromate test	-	+	-	+	+	+
Saponins	Foam Test	-	-	-	+	+	+
Phytosterols	Libermann's test	+	+	-	+	-	-
Carbohydrates	Molish test	-	-	-	+	-	-
Acid compounds	Litmus test	-	-	-	-	-	-
Glycoside	Borntragers test	-	-	-	+	-	+
Amino acids	Ninhydrin test	-	-	-	+	-	+
Proteins	Biuret test	-	-	-	+	-	+
Fixed oils & fats	Spot test	+	-	-	-	-	-

"+" indicates Presence and "-" indicates absence

i. *Characterization of isolated Phytoconstituents Stigmasterol*

White powder, $C_{29}H_{48}O$, MW 412.69. UV λ_{max} ($CHCl_3$) nm: 257; IR (KBr) ν_{max} 3418 (-OH), 2934, 2866, 2339, 1602, 1566, 1461, 1409, 1383, 1251, 1191, 1154, 1109, 1089, 1053, 1020, 791 cm^{-1}; ESMS m/z (%): 409.2, 395.3, 335, 161, 144, 121.1, 105.1, 97.1, 85.1, 69, 67.2, 65, 50.2; ^{1}H NMR (400 MHz, $CDCl_3$) δ ppm: 7.25 (1H, s, OH-2), 5.34-5.35 (1H, d), 5.12-5.18 (1H, m), 4.99-5.05 (1H, m), 3.48-3.56 (1H, m), 2.18-2.31 (2H, m), 1.93-2.09 (3H, m), 1.82-1.87 (2H, m), 1.66-1.75 (1H, m), 1.37-1.54 (13H, m), 1.05-1.31 (m, 7H), 0.99-1.01 (m, 8H), 0.90-0.98 (m, 2H), 0.78-0.85 (m, 9H), 0.66-0.70 (3H, t); ^{13}C NMR (400 MHz, $CDCl_3$) δ ppm: 140.85 (C-4), 138.31 (C-19), 129.40 (C-20), 121.72 (C-7), 77.34 (C-2), 71.86 (C-11), 56.95 (C-17), 56.09 (C-21), 51.29 (C-10), 50.29 (C-12), 42.41 (C-3), 42.30 (C-18), 40.46 (C-13), 39.77 (C-5), 37.35 (C-6), 36.59 (C-8), 32 (C-9), 31.96 (C-1), 31.91 (C-22), 31.77 (C-16), 28.91 (C-15), 25.41 (C-24), 24.41 (C-23), 21.24 (C-26), 21.14 (C-14), 21.06 (C-29), 19.42 (C-27), 19.03 (C-25), 12.23 (C-28). PC-01 was identified as Stigmasterol.

ii. *β-Sitosterol*

White powder, $C_{29}H_{50}O$, MW 414.70; UV λ_{max} ($CHCl_3$) nm: 251; IR (KBr) ν_{max} 3424, 2959, 2936, 2867, 1602, 1565, 1465, 1382, 1332, 1242, 1191, 1154, 1051, 779, 450, 432, 416cm^{-1}; ESMS m/z (%): 411.2, 397.3, 383.3, 311.2, 161.1, 81.2; ^{1}H NMR (400 MHz, $CDCl_3$) δ ppm: 7.30 (1H, s), 5.34-5.35 (1H, d), 4.98-5.19 (1H, m), 3.47-3.55 (1H, m), 2.19-2.31 (2H, m), 1.03-1.30 (9H, m), 1.00 (4H, s), 0.90-0.98 (4H, m), 0.76-0.86 (9H, m), 0.68-0.69 (3H, d), 1.94-2.07 (2H, m), 1.79-1.88 (4H, m); ^{13}C NMR (400 MHz, $CDCl_3$) δ ppm: 140.84 (C-4), 121.70 (C-7), 71.82 (C-2), 56.94 (C-11), 56.85 (C-17), 50.25 (C-10), 45.95 (C-21), 42.39 (C-7), 42.36 (C-3), 39.87 (C-13), 37.34 (C-5), 36.57 (C-6), 36.19 (C-18), 33.78(C-19), 32.15 (C-8), 31.99 (C-9), 31.97 (C-7), 30.39 (C-22), 26.28 (C-20), 25.90 (C-15), 25.40 (C-16), 24.40 (C-24), 23.2 (C-23), 21.17 (C-26), 21.06 (C-14) 21.06 (C-29), 19.32 (C-27), 19.34 (C-25), 12.11 (C-28). PC-02 was identified as β-Sitosterol.

iii. *Ursolic acid*

White powder, $C_{30}H_{48}O_3$, MW 456.7 ; UV λ_{max} (EtOH) nm: 203; IR (KBr) ν_{max} 3450, 2925, 2869, 2339, 1556, 1456, 1387, 1247, 1157, 822, 444, 433, 422, 415cm^{-1}; ESMS m/z (%): 455.2 $(M-1)^+$, 456.2, 457.3; ^{1}H NMR (400 MHz, DMSO) δ ppm: 11.91 (1H, s), 5.14 (1H, s), 4.27 (1H, s), 3.01 (1H, s), 2.51 (1H, s), 2.10-2.13 (1H, d) 1.85-1.93 (4H, t), 1.26-1.32 (4H, t), 1.05 (1H, s), 0.91-0.92 (8H, d), 0.88 (1H, s) 0.82-0.83 (4H, d), 0.76 (3H, s), 0.69 (4H,s); ^{13}C NMR (400 MHz, DMSO) δ ppm: 178.16 (C-29), 138.17 (C-12), 124.58 (C-13), 76.86 (C-2), 56.01 (C-4), 54.82 (C-18), 52.40 (C-11), 47.05 (C-10), 46.82 (C-17), 41.64 (C-9), 40.41 (C-3), 40.21 (C-22), 40 (C-6), 39.79 (C-5), 39.58 (C-19), 39.37 (C-8), 39.16 (C-20), 38.96 (C-1), 38.49 (C-15), 38.46 (C-16), 38.36 (C-23), 38.28 (C-24), 36.53 (C-14), 36.31 (C-30), 32.73 (C-7), 30.2 (C-28), 28.24 (C-26), 27.55 (C-27), 26.99 (C-15). PC-03 was identified as Ursolic acid.

iv. *Oleanolic acid*

White powder, $C_{30}H_{48}O_3$, MW 456.71; UV λ_{max} (EtOH) nm: 210; IR (KBr) ν_{max} 3443, 2941, 2862, 1694, 1602, 1566, 1462, 1388, 1364, 1304, 1273, 1208, 1185, 1161, 1093, 1028, 960, 791 cm^{-1}; ESMS m/z (%): 455.3, 456.2; 1H NMR (400 MHz, DMSO) δ ppm: 12 (1H, s), 5.16 (1H, s), 4.27 (1H, s), 3 (1H, s), 2.73-2.77 (1H, m), 1.88-1.95 (1H, s), 1.80-1.83 (2H, m), 1.58-1.70 (3H, m), 1.42-1.50 (8H, m), 1.23-1.38 (5H, m), 1.07-1.10 (4H, t), 0.98-1.01 (1H, m), 0.86-0.93 (14H, m), 0.72 (3H, s), 0.68 (5H, s); 13C NMR (400 MHz) 178.52 (C-28), 143.83 (C-12), 121.49 (C-13), 76.83 (C-2), 54.81 (C-4), 47.09 (C-11), 45.70 (C-10), 45.44 (C-22), 41.32 (C-17), 40.82 (C-22), 40.20 (C-18), 39.99 (C-12), 39.58 (C-9), 39.37 (C-6), 39.16 (C-3), 38.95 (C-5), 38.89 (C-8), 38.36 (C-19), 38.07 (C-21), 36.60 (C-1), 33.34 (C-29), 32.80 (C-30), 32.43 (C-16), 32.09 (C-14), 30.35 (C-23), 28.21 (C-24), 27.20 (C-7), 26.94 (C-26), 14.82 (C-27). PC-04 was identified as Oleanolic acid.

c) Hepatoprotective activity

Serum biochemical parameters are shown in Table 2. PCM administration induced destruction to hepatocytes confirmed by raised level of liver enzymes (ALT, AST, and ALP), total bilirubin and bilirubin as compared to control. Elevated levels of these enzymes are indicative of cellular damage and loss of functional integrity of hepatocytes. A single dose of PCM (2 g/kg) significantly higher ($P < 0.001$), elevated the ALT, AST, ALP, bilirubin and cholesterol levels (107.33, 100.33, 289, 0.10 and 75.23 units/mL) when compared to the normal animals (45.33, 51, 111.33, 0.08 and 39.63 units/mL) respectively indicating elevation in enzyme levels. Treatment of the rats with the MEAR (200mg/Kg, 400mg/kg), Oleanolic acid, Ursolic acid, β-Sitosterol and stigmasterol have decreased the enzyme levels in the range of 51 - 73 units/mL for ALT, 42 - 68 units/mL for AST, 100 – 151 units/mL for ALP and 0.04 – 0.06 units/mL for bilirubin, 44 – 58 units/mL for cholesterol which were found to be comparable to the enzyme levels (AST, ALT, ALP and TP) elevated by PCM induced rats. Standard drug Silymarin also reduced the enzyme levels in the range of 38.33, 41.67, 94, 0.04 and 40.3 units/mL, for ALT, AST, ALP, bilirubin and cholesterol levels respectively. Results for histopathological examination are given in Figures 2 & 3.

Table 2: Effects of pretreatment with Aralia racemosa L. extract, Oleanolic acid, Ursolic acid, β-Sitosterol and Stigmasterol on the serum levels of AST, ALT, ALP, Bilrubin, cholesterol and total proteins in PCM induced hepatotoxicity in rat.

Treatment groups and liver specific Variables	I (Normal Control: 0.5% Tween 80 1ml/kg b.wt)	II (Hepatotoxic Control: 0.5% Tween80 1ml/kg b.wt+PCM (2g/Kg b.wt)	III (MEAR 200mg/kg b.wt + PCM 2g/kg. b.wt)	IV (MEAR 400mg/kg b.wt +PCM 2g/kg. b.wt)	V (Oleanolic acid 20 mg/kg b.wt +PCM 2g/kg. b.wt)	VI (Ursolic acid 20 mg/kg b.wt +PCM 2g/kg. b.wt)	VII (β-Sitosterol 20 mg/Kg b.wt+PCM 2g/kg b.wt)	VIII (Stigmasterol 20 mg/Kg b.wt+PCM 2g/kg b.wt)	IX (Silymarin 25 mg/kg b. wt. + PCM 2 g/kg b. wt.)
AST (U/L)	51±4.36	100.33±10.6	68.67±4.93*	56.33±7.76*	51±5.56*	42.67±5.03 *	53.33±5.03*	44.33±4.04*	41.67±4.04*
ALT (U/L)	45.33±4.93	107.33±3.79	73.33±10.69*	61.67±6.51*	45±6.56*	42.67±3.05*	54.32±2.90*	51±3.60*	38.33±4.51*
ALP (U/L)	111.33±9.07	289±10.15	151.33±10.50*	126.7±6.11*	107.7±8.73*	100±8 *	117.7±9.2*	108.3±6.50 *	94±9.17*
Total Bilrubin (mg/dl)	0.08±0.01	0.10±0.02	0.05±0.02[b]	0.053±0.05[b]	0.05±0.01[b]	0.04±0.02[a]	0.06±0.02	0.05±0.01[b]	0.04±0.02[a]
Cholesterol (mg/dl)	39.63±4.15	75.23±3.22	58.2±2.38*	51.35±2.43*	48.3±1.98*	44.36±2.05*	51±3.72*	46.64±1.32*	40.31±2.32*
Total proteins (g/dl)	6.43±0.153	3.46±0.25	4.89±0.13*	5.06±0.07*	5.51±0.13*	5.68±0.151*	5.24±0.12*	5.51±0.14*	5.94±0.13*

All values expressed as mean±SEM; n=5 rat in each group, by one-way ANOVA followed by Tukey's Multiple Comparison Test.*, $p<0.001$ a, $p<0.01$, b, $p<0.05$ versus PCM treated group.

Silymarin is a well-established hepatoprotective drug able to reduce the elevated levels of liver enzymes in various drug-induced hepatotoxicity. The administration of test compounds raised the reduced level of total protein in the range of 4.89 – 5.51 units/mL and also decreased the elevated values of other enzymes as compared to toxicity value induced.

d) Histopathology

The histological examination of the liver section of the normal control group showed the normal architecture of normal liver histology i.e., hepatic central vein and sinusoids (Fig. 4A). The liver sections of rats treated with PCM alone showed prominent hepatic cell necrosis. (Fig. 4B). The liver section of rat treated with PCM and silymarin-treated groups preserves the almost normal structure of hepatocytes (Fig. 4I). In MEAR (200, 400 mg/Kg) treated groups, showing liver restoring to normalcy with little hepatic damage (Fig. 2C-D). In Oleanolic acid (20mg/Kg), Ursolic acid (20mg/Kg), β-Sitosterol (20 mg/kg) and Stigmasterol (20 mg/Kg) treated groups, showed complete restoration of necrosis with the normal architecture of hepatocytes (Fig 4E-H). (Fig. 4).

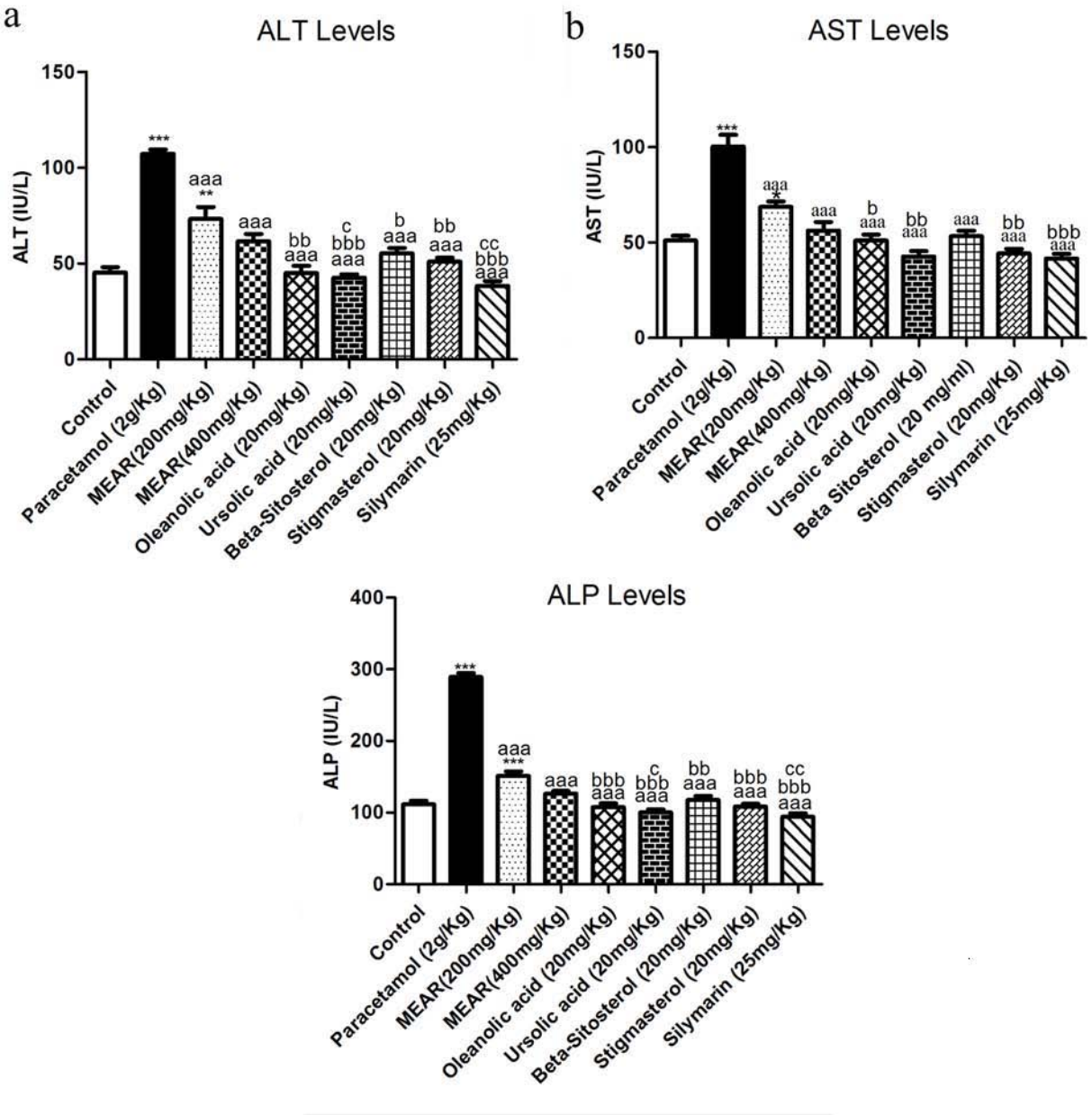

Fig. 2: Serum enzymic indices of liver toxicity in rats intoxicated with PCM and administered MEAR (200 and 400 mg/Kg), Oleanolic acid, Ursolic acid, β-Sitosterol and Stigmasterol. All values expressed as mean±SEM; n=5 rat in each group, by one-way ANOVA followed by Tukey's Multiple Comparison Test. (a)Alanine transaminase activity in all groups.***, $p<0.001$ versus control,**, $p<0.01$ versus control, aaa, $p<0.001$ versus PCM, bbb, $p<0.001$ versus MEAR (200mg/Kg), bb, $p<0.01$ versus PEAR (200mg/Kg), b, $p<0.05$ versus MEAR (200mg/Kg), cc, $p<0.01$ versus MEAR (400mg/Kg), c, $p<0.05$ versus MEAR (400 mg/Kg) (b) Aspartate transaminase activity in all groups.***, $p<0.001$ versus control, aaa, $p<0.001$ versus PCM, bb, $p<0.01$ versus MEAR (200mg/Kg), b, $p<0.05$ versus MEAR (200 mg/kg) (c) Alkaline phosphatase activity in all groups***, $p<0.001$ versus control,**, $p<0.01$ versus control, aaa, $p<0.001$ versus PCM, bbb, $p<0.001$ versus MEAR (200mg/Kg), bb, $p<0.01$ versus MEAR (200mg/Kg) cc, $p<0.01$ versus MEAR (400 mg/Kg).

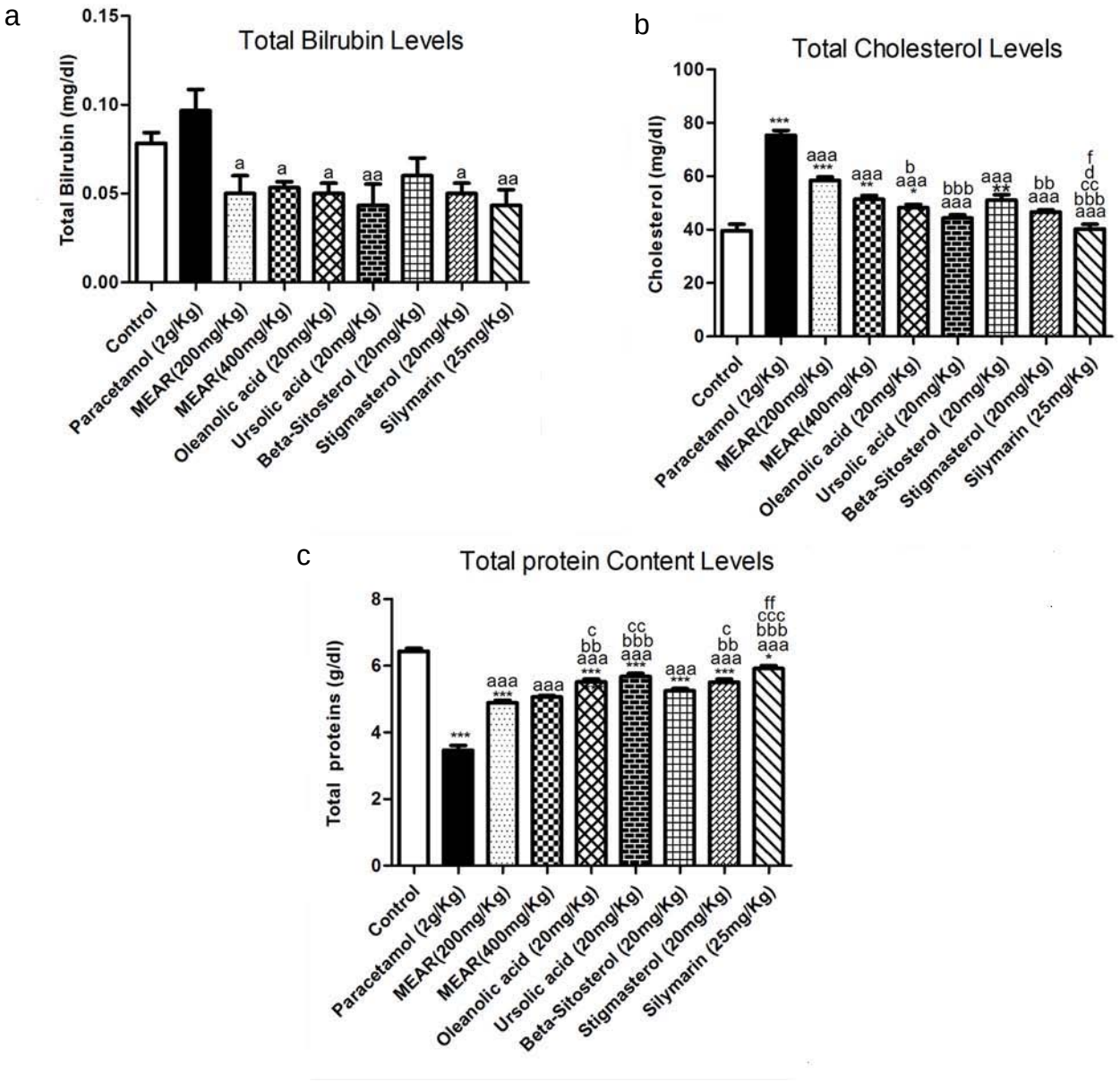

Fig. 3: Serum enzymic indices of liver toxicity in rats intoxicated with PCM and administered MEAR (200 and 400 mg/Kg), Oleanolic acid, Ursolic acid, β-Sitosterol and Stigmasterol. All values expressed as mean±SEM; n=5 rat in each group, by one-way ANOVA followed by Tukey's Multiple Comparison Test. (a)Serum bilrubin level in all groups. aa, $p<0.01$ versus PCM, a, $p<0.05$ versus PCM. (b) Serum cholesterol level in all groups.***, $p<0.001$ versus control,**, $p<0.01$ versus control,*, $p<0.05$ versus control, aaa, $p<0.001$ versus PCM, bbb, $p<0.001$ versus MEAR (200mg/Kg), bb, $p<0.01$ versus MEAR (200 mg/kg), b, $p<0.05$ versus MEAR (200mg/Kg), cc, $p<0.01$ versus MEAR(400mg/Kg), d, $p<0.05$ versus Oleanolic acid (20mg/Kg), f, $p<0.01$ versus β-Sitosterol (20 mg/kg). (c) Protein level in all groups.***, $p<0.001$ versus control,*, $p<0.05$ versus control, aaa, $p<0.001$ versus PCM, bbb, $p<0.001$ versus MEAR (200mg/Kg), bb, $p<0.01$ versus MEAR (200mg/Kg), ccc, $p<0.001$ versus MEAR (400mg/Kg), c, $p<0.05$ versus MEAR (400mg/kg), ff, $p<0.01$ versus β-Sitosterol (20 mg/kg).

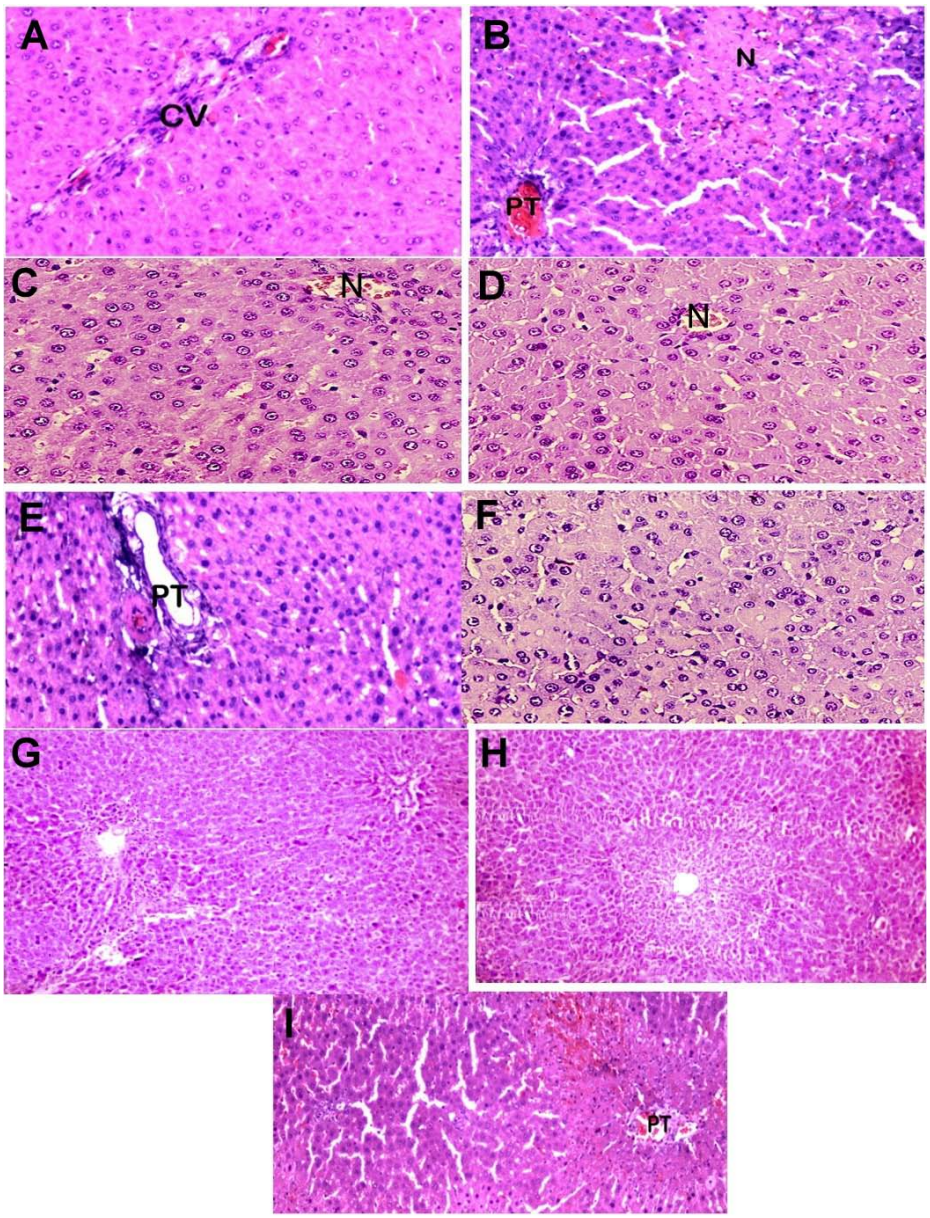

Fig. 4: Paraffin sections of liver stained by haematoxylin and eosin for histopathological changes. **(A)** Liver section of control group showing normal architecture of normal liver histology. **(B)** Hepatotoxic liver after treatment of PCM (2gm /Kg) **(C)** Liver section treated with PCM + MEAR (200 mg/kg) **(D)** Liver section treated with PCM and MEAR (400 mg/kg) **(E)** Liver section treated with PCM and Oleanolic acid (20 mg/kg) **(F)** Liver section treated with PCM and Ursolic acid (20 mg/kg) **(G)** Liver Section treated with PCM and β-Sitosterol (20mg/Kg) **(H)** Liver Section treated with PCM and Stigmasterol (20mg/Kg) **(I)** Liver Section treated with PCM and Silymarin (25mg/Kg).

PT = Portal Triad, CV = Central Vein, N = Necrosis.

IV. Discussion and Conclusion

Plant medicines play a significant role by their various formulations for the remedying of various diseases. A few are already examined and scientifically validated for their potentials. Here, we designed the experiments to examine the hepatoprotective activity of MEAR for their development into safe natural drug candidates.

PCM is extensively used being an antipyretic drug that is safe in therapeutic doses, however, could cause fatal hepatic damage in human beings and animal at higher toxic doses. Bioactivation of PCM by hepatic cytochrome P- 450 result in the formation of an extremely reactive and toxic metabolite N-acetyl-p-benzoquinone imine (NAPQI). NAPQI is generally detoxified through conjugation with reduced glutathione (GSH) to form the mercapturic acid that is eliminated by

urine. The toxic overdose associated with PCM impoverishes hepatic GSH content so free NAPQI binds covalently to cellular mitochondrial proteins that inhibit mitochondrial fatty acid -oxidation and leads to significant necrosis and apoptosis of hepatocytes (Chen, Krausz, Shah, Idle, & Gonzalez, 2009; Kiran et al., 2012). A distinct sign of hepatic injury is the leaking of cellular enzymes like ALT, AST, and ALP into plasma because of the disruption caused by the transport functions of hepatocytes. ALT is more specific to the liver, and it is a surpassing criterion for analyzing hepatic injury. Higher levels of AST signify the cellular exudation together with the diminished functional ability of cell membrane in the liver. Serum ALP is also regarding to liver cell damage. High concentration of ALP results in serious hepatic damage in PCM treated rats (Bhattacharyya et al., 2013). The liver is the key source of the majority of the serum proteins. Bilirubin is a product of heme within the reticuloendothelia system; its marked up in the blood stream could be adduced to over production, increased hemolysis, decreased conjugation or impaired bilirubin transport (Abirami, Nagarani, & Siddhuraju, 2015). Bilirubin is a recueil which is used to assess the normal functioning of the liver rather than the extent of the hepatocellular injury. Phytoconstituents including triterpenoids and flavonoids are well recognized for their antioxidant and hepatoprotective activities. Phytochemical analysis of methanolic extract of *A. racemosa* revealed the presence of tannins, volatile oils, flavonoids, saponins, triterpenes, and glycosides.

In ALT, PCM treated group demonstrated a sententious exaggeration ($P < 0.001$) in the values in comparison to control group. There was a significant abatement ($p< 0.001$) in the enzyme level of the treated groups i.e., III - IX in comparison to PCM treated group.

In AST, PCM treated group exhibited a significant increase ($P < 0.001$) in the values in comparison to control group. There was a significant change ($p< 0.001$) in the enzyme level reduction of the treated groups i.e., III - IX in comparison to PCM treated group.

In ALP, PCM treated group exhibited a significant increase ($P < 0.01$) in the values in comparison to control group. There was a significant change ($p< 0.001$) in the enzyme level reduction of the treated groups i.e., III- IX in comparison to PCM treated group.

In Cholesterol, PCM treated group exhibited a significant increase ($p< 0.001$) in the values in comparison to control group except in silymarin treated group. There was a significant reduction ($p<0.001$) in the enzyme level reduction of the treated groups i.e., III – IX in comparison to PCM treated group.

In the Total protein, PCM treated group exhibited a significant increase ($p< 0.001$) in the values in comparison to control group except in silymarin treated group with significance $p<0.05$. There was a significant reduction ($p<0.001$) in the enzyme level reduction of the treated groups i.e., III - IX in comparison to PCM treated group. (Figure 4).

Our results provided strong evidence that *A. racemosa* extracts significantly inhibited the acute liver toxicity induced by high doses of PCM in the rat, as shown by a decrease in serum liver enzyme activities (AST, ALT, and ALP) and bilirubin concentrations (Tables 2). Moreover, the liver morphology and histopathology findings confirm the protective activity of this extract against the PCM induced liver damage as it is evident by the reversal of centrilobular necrosis in hepatic parenchyma by *A. racemosa* administration. Thus, as shown in Figures 2C and 2D, only mild inflammation was observed. Although this protective effect was dose-dependent, there was no significant difference between doses of 200 and 400 mg/ Kg of *A. racemosa* methanolic extract. Despite the fact that *A. racemosa* extracts significantly reduced ALT and AST levels in groups III and IV can't completely restore these biochemical parameters to the normal values. Moreover, group V - IX that received Oleanolic acid, Ursolic acid, β-Sitosterol, and Stigmasterol showed significant differences with negative control group based on biochemical parameters (AST, ALT, ALP and bilirubin) and histopathological findings.

In conclusion, the results of the study demonstrate that methanolic extract of *A. racemosa* root possesses hepatoprotective activity against PCM induced liver injury in the rat. This property was attributed to the presence of triterpenes i.e., ursolic acid and Oleanolic acid which can be proven to normalize the disturbed antioxidant status possibly by maintaining the levels of glutathione through by inhibiting the production of malondialdehyde or might be because of inhibition of toxicant activation and the enhancement of body defense system. The hepatoprotection afforded by sterols i.e., β-Sitosterol and Stigmasterol were associated with the enhancement in mitochondrial glutathione redox status, possibly with the glutathione reductase-mediated improvement in mitochondrial glutathione redox cycling. Thus, these kinds of triterpene and phytosterols serve as a potential mitohormetic agent for the prevention of oxidative stress evoked in the liver.

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Physicochemical, Volatile Organic Composition, Phenolic, Flavonoid and Ascorbic Acid Contents, Antioxidant, Anti-Arthritic and Anti-Inflammatory Properties of *Cocos nucifera* Juice

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Abstract- Various parts of *Cocos nucifera* are locally used for treatment of diseases and production of some foods and beverages for man and animals. This study examined the physicochemical properties, phytochemical and multi therapeutic potentials of juice of *C. nucifera* from Nigeria. These were measured using GC-MS, pH meter, specific gravity, UV-Vis spectrometry, Folin-Ciocalteu's, aluminium chloride, DPPH, PTAC and egg albumin methods respectively. GC-MS analysis revealed the presence of nitroisobutylglycerol as the most abundant volatile organic compounds in the juice. The pH, clarity, turbidity, TPC, TFC and TAA were 5.09, 1.34, 1.07, 2,261.5±0.00 μgmg^{-1} GAE, 20.00±0.0 μgmg^{-1} QE and 66.75±0.00 μgmg^{-1} AAE, respectively. The antioxidant IC_{50} and AAI values of the juice were 0.25 mgml^{-1} and 160 and it was capable of scavenging free radicals at a range between 33.09- 76.26%. The TAC was 645.38±0.00 μgmg^{-1} AAE. The protein denaturation inhibition capacity was at the range between 42.0-73.4%. Therefore, the juice of *C. nucifera* could be used as multi therapeutic agent.

GJMR-B Classification: NLMC Code: QV 55

Physicochemical, Volatile Organic Composition, Phenolic, Flavonoid and Ascorbic Acid Contents, Antioxidant, Anti-Arthritic and Anti-Inflammatory Properties of *Cocos nucifera* Juice

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I. Introduction

Natural products are good drug leads and phytochemical probes to explore mechanisms associated with infection of diseases (Kingston, 2011; Ibekwe and Ameh, 2014). Numerous plant polyphenols reportedly afford multiple health benefits. They are regarded as healthy food and nutrient sources because of their many beneficial components (Voravuthikunchai and Howe, 2014; Zhang *et al*., 2016). Secondary metabolites in the natural products are considered to be responsible for positive health outcomes (Gechev *et al*., 2014). Particularly, it is widely noted that plants produce a great deal of antioxidants to combat the oxidative stress induced by oxygen and light in the natural environment (Cartea *et al*., 2011; Li *et al*., 2016). Natural products possess antimicrobial and antioxidant activities responsible for the prevalence of dermatophytosis (Thebo *et al*., 2016). Many approved therapeutics and drugs are derived from natural sources (Cragg and Newman, 2013; Lahlou, 2013).

Cocos nucifera Linn commonly known as coconut is an important fruit tree in the tropical regions and the fruit can be made into a variety of foods and beverages (Yong *et al*., 2009). *C. nucifera* is an important member of the family *Arecaceae*. The juice of *C. nucifera* has the ability to prevent diseases and sickness. This is due to the free radical scavenging abilities of the antioxidant phytochemicals in it. *C. nucifera* has long been used in traditional medicine for different kind of illness and almost all parts have their uses. The juice inside the fruit is sterile but when it is extracted and exposed to air, it becomes subjected to quick oxidation and microbial contamination leading to depletion of nutrients and spoilage (Matsui *et al*., 2008; Queiroz *et al*., 2008; Jean *et al*., 2009; Nakono *et al*., 2012; Adubofuor *et al*., 2016). Juice of *C. nucifera* is one of the natural food products to quench thirst and easily available in most of the countries. Both water and meat of coconut refresh the body by providing nutritious content. Traditionally, it has been used to protect the body against infection by dangerous diseases. It has been found to improve digestion and hasten the absorption of nutrients including vitamins, minerals, and amino acids. Recently, the health and medicinal uses of *C. nucifera* products get research interest because it contains several metabolites such as sugars, proteins, free amino acids, vitamins, minerals and growth promoting factors (Reddy and Lakshmi, 2014). The juice of *C. nucifera* contains many enzymes including acid phosphatase, catalase, dehydrogenase, diastase, peroxidase and RNA polymerase. Juice of *C. nucifera* is locally consumed fresh, directly from the fruit. (Adubofuor *et al*., 2016). This study aimed at evaluations of physiochemical, phytochemical and therapeutic efficacies of *C. nucifera* juice.

II. Materials and Methods

The fruit of *C. nucifera* was gotten from Ota, Ogun State, Nigeria and the juice was collected and then stored in vial at 5 °C temperature to prevent contamination.

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a) Determination of Clarity and Turbidity

Clarity and turbidity of the juice was determined by measuring the absorbance at 525 and 660 nm respectively using a UV-Vis spectrophotometer (Surajbhan *et al*., 2012).

b) Colour Determination

Colour of the juice was determined by physical observation in day light (Barkatullah *et al*., 2012).

c) Odour Determination

Odour of the juice was determined by organoleptic evaluation (Aloko *et al*., 2017).

d) Determination of pH

The pH of the juice of *C. nucifera* was determined immediately after extraction at room temperature using digital pH meter (Paz *et al*., 2016).

e) Determination of Specific Gravity (SG)

A clean specific gravity bottle was weighted (W_0). Then the bottle was filled to the brim with water and stopper was inserted. The water on the stopper and bottle were carefully wiped off and reweighed (W_1). Same process was repeated, but using juice samples instead of water and weighted again (W_2). The specific gravity of the juice was calculated using the formula below.

Specific gravity = $(W_2-W_0)/(W_1-W_0)$

Where:

W_0 = Weight of empty specific gravity bottle
W_1 = Weight of water + specific gravity bottle
W_2 = Weight of test sample + specific gravity bottle.

f) GC-MS Analysis

The juice of *C. nucifera* dissolved in methanol was analysed using Shimadzu GC-MS-QP2010 Plus (Japan). The separations were carried out using a Restek Rtx-5MS fused silica capillary column (5%-diphenyl-95%-dimethylpolysiloxane) of 30 m× 0.25 mm internal diameter (di) and 0.25 mm in film thickness. The conditions for analysis were set as follows; column oven temperature was programmed from 60-280 °C (temperature at 60 °C was held for 1.0 min, raised to 180 °C for 3 min and then finally to 280 °C held for 2 min); injection mode, Split ratio 41.6; injection temperature, 250 °C; flow control mode, linear velocity (36.2 cm/sec); purge flow 3.0 ml/min; pressure, 56.2 kPa; helium was the carrier gas with total flow rate 45.0 ml/min; column flow rate, 0.99 ml/min; ion source temperature, 200 °C; interface temperature, 250 °C; solvent cut time, 3.0 min; start time 3.5 min; end time, 24.0 min; start m/z, 50 and end m/z, 700. Detector was operated in EI ionization mode of 70 eV. Components were identified by matching their mass spectra with those of the spectrometer data base using the NIST computer data bank, as well as by comparison of the fragmentation pattern with those reported in the literature (Ololade *et al*., 2017).

g) Determination of Total Phenolic Content (TPC)

The TPC of the juice of *C. nucifera* was determined using Folin-Ciocalteau method. 1 ml of juice was mixed with 1 ml of 10% Folin-Ciocalteu reagent in distilled water and then neutralized with 4 ml of 7.5% sodium carbonate solution. The sample was maintained at room temperature for 3 hrs with periodical mixing, the absorbance at 760 nm was measured using UV-vis spectrophotometer. Gallic acid was used as a reference and for the calibration curve; result was expressed in micrograms per gram of gallic acid equivalent (Vasudevarao and Sravanthi, 2017).

h) Total Flavonoid Concentration (TFC)

The total flavonoid content of the juice of *C. nucifera* was determined by spectrophotometry, using aluminium chloride method. Briefly, 1.0 ml of the juice, 0.10 ml of 10% aluminium chloride, 0.10 ml of sodium acetate (1 M) and 2.80 ml of distilled water. After incubation for 40 min, absorbance was measured at 415 nm using a UV-Vis-spectrophotometer. To calculate the concentration of flavonoids, we prepared a calibration curve using quercetin as standard. The index of TFC concentration is expressed as quercetin equivalents (QE) in μg per mg of juice. All assays were carried out in triplicate (Formagio *et al*., 2015).

i) Determination of Total Ascorbic acid content (TAA)

1 ml of the juice was added to 1.0 ml 2,4-dinitrophenylhydrazine (2,4-DNPH). It was allowed to stand for 30 min. and the absorbance was read in triplicate at 515 nm, using distilled water as blank. Ascorbic acid was used as a reference and for the calibration curve; result was expressed in milligram per gram of ascorbic acid equivalent (Benites *et al*., 2015).

j) Determination of Free Radical Scavenging and Antioxidant Activities

i. *In vitro 2,2'- Diphenyl-1-picryl-hydrazyl Assay*

The antioxidant and free radical scavenging of the juice of *C. nucifera* was measured by using DPPH. Briefly, the reaction mixture of 2.0 ml; consist of 1.0 ml of DPPH in methanol (0.004%) and 1.0 ml of various concentrations of juice. Then incubated for 30 min. in dark, and the absorbance was measured at 517 nm. The control was prepared by DPPH and methanol in place of sample. In this assay, the positive control is ascorbic acid. The percentage of inhibition can be calculated using the formula:

$$I\% = [(A_{blank} - A_{juc})/A_{blank}] \times 100$$

Where: A_{blank} is the absorbance of blank solution and A_{juc} is the absorbance of the juice. The dose response curve was plotted and IC_{50} value for the juice and the standard were calculated (Ololade *et al*., 2016).

Antioxidant Activity Index: The antioxidant activity index (AAI) was calculated as:

$$AAI = [DPPH\ initial\ concentration]/[IC_{50}]$$

AAI was classified as weak, when AAI < 0.5, moderate, when AAI ranged between 0.5-1.0, strong, when AAI ranged between 1.0-2.0, and very strong, when AAI > 2.0 (Arulpriya and Lalitha, 2014).

ii. *Phosphomolybdate Total Antioxidant Capacity (PTAC) Assay*

The PTAC of the juice of *C. nucifera* was determined with phosphomolybdenum using ascorbic acid as the standard. An aliquot of 1.0 ml of juice solution is combined with 1.0 ml of reagent (0.6 M sulphuric acid, 28 μM sodium phosphate and 4 μM ammonium molybdate). The tubes were capped and incubated in a boiling water bath at 95 ^{0}C for 90 min. after the samples had cooled to room temperature, the absorbance of the aqueous solution of each were measured at 695 nm 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 the rest of the samples. The total antioxidant capacity was expressed as equivalents of ascorbic acid (Bulus *et al*., 2017).

In-vitro Anti-Arthritic and Anti-Inflammatory Activities of the Juice on Inhibition of Protein Denaturation (Egg Albumin Assay): in vitro anti-arthritic/anti-inflammatory activity of the juice was evaluated against protein denaturation method using fresh hen's egg albumin. About 5 ml reaction mixtures (0.2 ml of egg albumin, 2.8 ml of phosphate buffered saline (PBS, pH 6.4) add 2 ml of test sample at 1000, 500, 250 and 125 μgml^{-1}). Distilled water with same volume (0.2 ml) was used as control. The mixtures were incubated at 37 ^{o}C in BOD incubator for about 15 min. followed by heating at 70 ^{o}C for 5 min. After cooling to the room temperature, absorbance was measured spectrophotometrically at 660 nm using vehicle as blank. Aspirin (3000 μgml^{-1}) was used as reference drug. The inhibition percentage of protein denaturation was calculated using the following formula:

$$\%\ inhibition = 100 \times (Vt/Vc) - 1$$

Where:

V_t = absorbance of test sample,
V_c = absorbance of control.

The drug concentration for 50% inhibition (IC_{50}) was determined by plotting percentage inhibition with respect to control against treatment concentration. (Smitha *et al*., 2017).

III. Results and Discussion

a) Physicochemical Properties of the Juice of C. nucifera

Determination of different physicochemical properties showed the practical importance and provides bases for suitability, consumption, utility, nutritional and physical qualities of the natural juice of *C. nucifera* in daily life (Angaye and Maduelosi, 2015). Physicochemical properties of the juice such as colour, odour, pH, clarity, turbidity, specific gravity (Table 1) showed the quality of the juice of *C. nucifera* from Nigeria.

b) Colour and Odour of the Juice of C. nucifera

The colour of the fresh juice of *C. nucifera* was milky in nature with sweet aromatic odour.

c) Clarity and Turbidity of the Juice of C. nucifera

The clarity and turbidity of the juice of *C. nucifera* were determined as 0.74 and 0.66 respectively using UV-Vis spectrophotometer.

d) pH of the Juice of C. nucifera

The pH value of the *C. nucifera* juice was 5.09; which was within the standard limit (pH 3.40–6.10) that insures freshness of the juice (El-Sohaimy *et al*., 2015). The acidity of juice might be due of present organic acid in the juice, which is responsible for important characteristics of juice: flavour and stability against microbial spoilage and this may confer longer keeping quality of the juice (Nadzirah *et al*., 2012; Offia-Olua and Ekwunife, 2015). Furthermore, it might also indicate that the juice of *C. nucifera* have high content of minerals. pH is a very important parameter in the conduct of fermentation. A pH of 4 is the optimum for the growth of fermentative yeast. This also inhibits the development of undesirable microbial flora (Ahoussi *et al*., 2015; Walker and Stewart, 2016).

e) Specific Gravity of the Juice of C. nucifera

The specific gravity of the juice of *C. nucifera* was 1.01 (Table 1). This was in line with the amount stated for beverages (including soft drinks and juices) and fruit drinks (low calories and undiluted) as 1.01-1.03. The more sugar present in a juice, the denser the juice becomes. Juice is low dense foods because of its high water content, which provides high volume and weight. To stay within low density guidelines, it is important to either consume natural fruit juice that has not been dehydrated than to eat processed fruit juice that contains added sugar (Swinburn *et al*., 2004; Ledikwe *et al*., 2006; Slavin and Lloyd, 2012; Babajide *et al*., 2013).

Table 1: Colour, Odour, Turbidity, pH, and Specific Gravity of the Juice of *C. nucifera*

Colour	Odour	Clarity	Turbidity	pH	SG
milky	Sweet aromatic smell	0.74	0.66	5.09	1.01

f) Organic Composition

GC-MS analysis revealed the presence of nitroisobutylglycerol ($C_4H_9NO_5$) as the most abundant volatile organic composition of the juice. The mass spectrum of the compound with retention time 14.942 and retention index 1444 gave 8 major peaks (m/z) at 27, 29, 31, 55, 57, 73, 85 and 86. Nitroisobutylglycerol is a low molecular weight (151) medicinal compound with oxytocin-induced, antioxidant, anti-staphylococcal activities (Rane and Anusha, 2012).

g) Total Phenolic Content, Total Flavonoid Contents and Total Ascorbic Acid

The TPC, TFC and TAA analyses of the investigated juice of *C. nucifera* showed the presence of high amount phenolic, flavonoid compounds and ascorbic acid (Table 2). Natural phenolic compounds and ascorbic acid play many significant roles in human health as evident from their therapeutic properties (Dimitros, 2006; Ansari *et al*., 2013; Dzialo *et al*., 2016). Plants consumed by humans may contain thousands of different amounts of ascorbic acid, phenolic and flavonoid components (Saxena *et al*., 2013; Kasote *et al*., 2015; Zhang *et al*., 2015). The effect of dietary ascorbic acid and phenolics is currently of great interest due to their antioxidative and possible anticarcinogenic activities (Nahak *et al*., 2014; Pereira *et al*., 2009). Ascorbic acid, Phenolic and flavonoid compounds are chain breaking antioxidant, free radicals scavenger and quenchers of singlet oxygen formation in the process of formation of intracellular substances throughout the body (Mitra and Uddin, 2014; Ozcan *et al*., 2014). Moreover, ascorbic acid, phenolic and flavonoid components play important roles in the control of cancer and other human diseases (Ghasemzadeh and Ghasemzadeh, 2011).

Table 2: TPC, TFC and TAA of the Juice of *C. nucifera*

TPC	TFC	TAA
2,261.5±0.00 μgmg^{-1} GAE	20.0±0.00 μgmg^{-1} QE	66.75±0.00 μgmg^{-1} AAE

Data are presented as the mean value ± S.D. of triplicate

h) Free Radical Scavenging and Antioxidant Potentials

The percentage inhibitions of the juice at various concentrations (1000, 750, 500 and 250 μgml^{-1}) were 76.26, 71.22, 71.00 and 33.09% respectively; while the IC_{50} value was found to be 0.25 $mgml^{-1}$ in comparison to ascorbic acid with IC_{50} value of 9.0 μgml^{-1}.

i) Phosphomolybdate Total Antioxidant Capacity (PTAC)

The PTAC of the juice of *C. nucifera* was 645.38±0.00 μgmg^{-1} AAE (Table 3). The measure of the ability of natural products to delay oxidative stress in a controlled system is defined as total antioxidant capacity (Apak *et al*., 2016; Zhang *et al*., 2016; Pieme *et al*., 2017; Tyagi and Agarwal, 2017). The juice showed high antioxidant potential and this can be related to the high amounts of ascorbic acid, flavonoids and phenolic compounds in juice. Antioxidant play definite roles in many pathological conditions and they are known to fight against these free radicals and protects body from various diseases (Aprioku *et al*., 2013; Lone *et al*., 2013). Their mechanism of action is either by scavenging the reactive oxygen species or protecting the antioxidant defence mechanisms (Birben *et al*., 2012). The total antioxidant potential is a relevant tool for investigating the relationship between dietary antioxidants and pathologies induced by the oxidative stress (Pisoschi and Negulescu, 2011).

Table 3: Antioxidant Properties of the Juice of *C. nucifera*

Juice and Reference Drug	DPPH IC_{50} $mgml^{-1}$	AAI	PTAC μgmg^{-1} AAE
Juice	0.25	160	645.38±0.00

j) In-vitro Anti-Arthritic and Anti-Inflammatory Potential

The juice of *C. nucifera* possesses potentially useful anti-arthritic and anti-inflammatory activities at the doses tested. The juice exhibited significant inhibition of egg albumins denaturation of protein between 42.0-73.4% at concentrations between 125-1000 μgml^{-1} and with the IC_{50} value of 6.0 μgml^{-1}. The result was comparable to that of standard drug aspirin (89.4%) (Table 4). The juice showed inhibition of heat-induced protein (albumin) denaturation and prominent effects on protein denaturation was produced. Natural products that can prevent protein denaturation would be very useful for the development of anti-arthritic and anti-inflammatory drugs (Sowjanya *et al*., 2013; Janakiraman and Parameswari, 2014; Obaseki *et al*., 2016). Therefore, the juice is a promising anti-arthritic agent of natural origin in the treatment of inflammatory disorders. It shows that the juice is capable of reducing the production of auto-antigen which indirectly reduces the protein denaturation and hence alleviate arthritis (Alamgeer *et al*., 2017; Boddupally *et al*., 2017). Protein denaturation is one of the leading causes of inflammatory as well as arthritic diseases, which led to production of auto antigens, progressing to certain rheumatic diseases (Jayaprakasam and Ravi, 2012; Pashikanti *et al*. 2014; Elisha *et al*., 2016; Mahabaland

Kaliwal, 2017). The main mechanism involved in protein denaturation is characterized by changes or alterations in hydrophobic, electrostatic, hydrogen and disulphide bonding among the protein molecules (Zavodszky *et al*., 2001; Sangeetha and Vidhya, 2016).

Table 4: Egg Albumin Anti-Arthritic/Anti-Inflammatory Activity of the Juice of *C. nucifera* and Reference Drug

Conc. μgml^{-1}	% Inhibition	IC_{50} μgml^{-1}	% Inhibition of Aspirin 3000 μgml^{-1}
1000	73.4		
500	71.4	6.0	89.4
250	48.0		
125	42.0		

IV. Conclusion

The results of this study showed that the juice of *C. nucifera* can be an accessible source of promising therapeutic agents that can be used in Combating some infectious diseases caused. The study showed the presence of significant antioxidant, anti-arthritic anti-inflammatory activities of the juice. The activities were due to the presence of pharmacologically active phytochemicals in the juice. Fresh juice of *C. nucifera* is a fluid that could be consumed for health, refreshment and nutritional purposes. Therefore the juice can include the production and commercialization of foods and drugs.

Conflict of Interest Statement: The authors declared that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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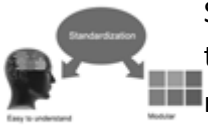

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1. GENERAL

Before submitting your research paper, one is advised to go through the details as mentioned in following heads. It will be beneficial, while peer reviewer justify your paper for publication.

Scope

The Global Journals Inc. (US) welcome the submission of original paper, review paper, survey article relevant to the all the streams of Philosophy and knowledge. The Global Journals Inc. (US) is parental platform for Global Journal of Computer Science and Technology, Researches in Engineering, Medical Research, Science Frontier Research, Human Social Science, Management, and Business organization. The choice of specific field can be done otherwise as following in Abstracting and Indexing Page on this Website. As the all Global

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Complete support for both authors and co-author is provided.

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Based on potential and nature, the manuscript can be categorized under the following heads:

Original research paper: Such papers are reports of high-level significant original research work.

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Research articles: These are handled with small investigation and applications

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The recommended size of original research paper is less than seven thousand words, review papers fewer than seven thousands words also.Preparation of research paper or how to write research paper, are major hurdle, while writing manuscript. The research articles and research letters should be fewer than three thousand words, the structure original research paper; sometime review paper should be as follows:

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(b) A brief Summary, "Abstract" (less than 150 words) containing the major results and conclusions.

(c) Up to ten keywords, that precisely identifies the paper's subject, purpose, and focus.

(d) An Introduction, giving necessary background excluding subheadings; objectives must be clearly declared.

(e) Resources and techniques with sufficient complete experimental details (wherever possible by reference) to permit repetition; sources of information must be given and numerical methods must be specified by reference, unless non-standard.

(f) Results should be presented concisely, by well-designed tables and/or figures; the same data may not be used in both; suitable statistical data should be given. All data must be obtained with attention to numerical detail in the planning stage. As reproduced design has been recognized to be important to experiments for a considerable time, the Editor has decided that any paper that appears not to have adequate numerical treatments of the data will be returned un-refereed;

(g) Discussion should cover the implications and consequences, not just recapitulating the results; conclusions should be summarizing.

(h) Brief Acknowledgements.

(i) References in the proper form.

Authors should very cautiously consider the preparation of papers to ensure that they communicate efficiently. Papers are much more likely to be accepted, if they are cautiously designed and laid out, contain few or no errors, are summarizing, and be conventional to the approach and instructions. They will in addition, be published with much less delays than those that require much technical and editorial correction.

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Metric SI units are supposed to generally be used excluding where they conflict with current practice or are confusing. For illustration, 1.4 l rather than 1.4 × 10-3 m3, or 4 mm somewhat than 4 × 10-3 m. Chemical formula and solutions must identify the form used, e.g. anhydrous or hydrated, and the concentration must be in clearly defined units. Common species names should be followed by underlines at the first mention. For following use the generic name should be constricted to a single letter, if it is clear.

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A major linchpin in research work for the writing research paper is the keyword search, which one will employ to find both library and Internet resources.

One must be persistent and creative in using keywords. An effective keyword search requires a strategy and planning a list of possible keywords and phrases to try.

Search engines for most searches, use Boolean searching, which is somewhat different from Internet searches. The Boolean search uses "operators," words (and, or, not, and near) that enable you to expand or narrow your affords. Tips for research paper while preparing research paper are very helpful guideline of research paper.

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- It may take the discovery of only one relevant paper to let steer in the right keyword direction because in most databases, the keywords under which a research paper is abstracted are listed with the paper.
- One should avoid outdated words.

Keywords are the key that opens a door to research work sources. Keyword searching is an art in which researcher's skills are bound to improve with experience and time.

Numerical Methods: Numerical methods used should be clear and, where appropriate, supported by references.

Acknowledgements: Please make these as concise as possible.

References

References follow the Harvard scheme of referencing. References in the text should cite the authors' names followed by the time of their publication, unless there are three or more authors when simply the first author's name is quoted followed by et al. unpublished work has to only be cited where necessary, and only in the text. Copies of references in press in other journals have to be supplied with submitted typescripts. It is necessary that all citations and references be carefully checked before submission, as mistakes or omissions will cause delays.

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Acrobat Reader will be required in order to read this file. This software can be downloaded

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TECHNIQUES FOR WRITING A GOOD QUALITY RESEARCH PAPER:

1. Choosing the topic: In most cases, the topic is searched by the interest of author but it can be also suggested by the guides. You can have several topics and then you can judge that in which topic or subject you are finding yourself most comfortable. This can be done by asking several questions to yourself, like Will I be able to carry our search in this area? Will I find all necessary recourses to accomplish the search? Will I be able to find all information in this field area? If the answer of these types of questions will be "Yes" then you can choose that topic. In most of the cases, you may have to conduct the surveys and have to visit several places because this field is related to Computer Science and Information Technology. Also, you may have to do a lot of work to find all rise and falls regarding the various data of that subject. Sometimes, detailed information plays a vital role, instead of short information.

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3. Think Like Evaluators: If you are in a confusion or getting demotivated that your paper will be accepted by evaluators or not, then think and try to evaluate your paper like an Evaluator. Try to understand that what an evaluator wants in your research paper and automatically you will have your answer.

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7. Use right software: Always use good quality software packages. If you are not capable to judge good software then you can lose quality of your paper unknowingly. There are various software programs available to help you, which you can get through Internet.

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15. Use of direct quotes: When you do research relevant to literature, history or current affairs then use of quotes become essential but if study is relevant to science then use of quotes is not preferable.

16. Use proper verb tense: Use proper verb tenses in your paper. Use past tense, to present those events that happened. Use present tense to indicate events that are going on. Use future tense to indicate future happening events. Use of improper and wrong tenses will confuse the evaluator. Avoid the sentences that are incomplete.

17. Never use online paper: If you are getting any paper on Internet, then never use it as your research paper because it might be possible that evaluator has already seen it or maybe it is outdated version.

18. Pick a good study spot: To do your research studies always try to pick a spot, which is quiet. Every spot is not for studies. Spot that suits you choose it and proceed further.

19. Know what you know: Always try to know, what you know by making objectives. Else, you will be confused and cannot achieve your target.

20. Use good quality grammar: Always use a good quality grammar and use words that will throw positive impact on evaluator. Use of good quality grammar does not mean to use tough words, that for each word the evaluator has to go through dictionary. Do not start sentence with a conjunction. Do not fragment sentences. Eliminate one-word sentences. Ignore passive voice. Do not ever use a big word when a diminutive one would suffice. Verbs have to be in agreement with their subjects. Prepositions are not expressions to finish sentences with. It is incorrect to ever divide an infinitive. Avoid clichés like the disease. Also, always shun irritating alliteration. Use language that is simple and straight forward. put together a neat summary.

21. Arrangement of information: Each section of the main body should start with an opening sentence and there should be a changeover at the end of the section. Give only valid and powerful arguments to your topic. You may also maintain your arguments with records.

22. Never start in last minute: Always start at right time and give enough time to research work. Leaving everything to the last minute will degrade your paper and spoil your work.

23. Multitasking in research is not good: Doing several things at the same time proves bad habit in case of research activity. Research is an area, where everything has a particular time slot. Divide your research work in parts and do particular part in particular time slot.

24. Never copy others' work: Never copy others' work and give it your name because if evaluator has seen it anywhere you will be in trouble.

25. Take proper rest and food: No matter how many hours you spend for your research activity, if you are not taking care of your health then all your efforts will be in vain. For a quality research, study is must, and this can be done by taking proper rest and food.

26. Go for seminars: Attend seminars if the topic is relevant to your research area. Utilize all your resources.

27. Refresh your mind after intervals: Try to give rest to your mind by listening to soft music or by sleeping in intervals. This will also improve your memory.

28. Make colleagues: Always try to make colleagues. No matter how sharper or intelligent you are, if you make colleagues you can have several ideas, which will be helpful for your research.

29. Think technically: Always think technically. If anything happens, then search its reasons, its benefits, and demerits.

30. Think and then print: When you will go to print your paper, notice that tables are not be split, headings are not detached from their descriptions, and page sequence is maintained.

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32. Never oversimplify everything: To add material in your research paper, never go for oversimplification. This will definitely irritate the evaluator. Be more or less specific. Also too, by no means, ever use rhythmic redundancies. Contractions aren't essential and shouldn't be there used. Comparisons are as terrible as clichés. Give up ampersands and abbreviations, and so on. Remove commas, that are, not necessary. Parenthetical words however should be together with this in commas. Understatement is all the time the complete best way to put onward earth-shaking thoughts. Give a detailed literary review.

33. Report concluded results: Use concluded results. From raw data, filter the results and then conclude your studies based on measurements and observations taken. Significant figures and appropriate number of decimal places should be used. Parenthetical remarks are prohibitive. Proofread carefully at final stage. In the end give outline to your arguments. Spot out perspectives of further study of this subject. Justify your conclusion by at the bottom of them with sufficient justifications and examples.

34. After conclusion: Once you have concluded your research, the next most important step is to present your findings. Presentation is extremely important as it is the definite medium though which your research is going to be in print to the rest of the crowd. Care should be taken to categorize your thoughts well and present them in a logical and neat manner. A good quality research paper format is essential because it serves to highlight your research paper and bring to light all necessary aspects in your research.

Informal Guidelines of Research Paper Writing

Key points to remember:

- Submit all work in its final form.
- Write your paper in the form, which is presented in the guidelines using the template.
- Please note the criterion for grading the final paper by peer-reviewers.

Final Points:

A purpose of organizing a research paper is to let people to interpret your effort selectively. The journal requires the following sections, submitted in the order listed, each section to start on a new page.

The introduction will be compiled from reference matter and will reflect the design processes or outline of basis that direct you to make study. As you will carry out the process of study, the method and process section will be constructed as like that. The result segment will show related statistics in nearly sequential order and will direct the reviewers next to the similar intellectual paths throughout the data that you took to carry out your study. The discussion section will provide understanding of the data and projections as to the implication of the results. The use of good quality references all through the paper will give the effort trustworthiness by representing an alertness of prior workings.

Writing a research paper is not an easy job no matter how trouble-free the actual research or concept. Practice, excellent preparation, and controlled record keeping are the only means to make straightforward the progression.

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Specific editorial column necessities for compliance of a manuscript will always take over from directions in these general guidelines.

To make a paper clear

· Adhere to recommended page limits

Mistakes to evade

- Insertion a title at the foot of a page with the subsequent text on the next page
- Separating a table/chart or figure - impound each figure/table to a single page
- Submitting a manuscript with pages out of sequence

In every sections of your document

· Use standard writing style including articles ("a", "the," etc.)

· Keep on paying attention on the research topic of the paper

· Use paragraphs to split each significant point (excluding for the abstract)

· Align the primary line of each section

· Present your points in sound order

· Use present tense to report well accepted

· Use past tense to describe specific results

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· Shun use of extra pictures - include only those figures essential to presenting results

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Choose a revealing title. It should be short. It should not have non-standard acronyms or abbreviations. It should not exceed two printed lines. It should include the name(s) and address (es) of all authors.

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The summary should be two hundred words or less. It should briefly and clearly explain the key findings reported in the manuscript--must have precise statistics. It should not have abnormal acronyms or abbreviations. It should be logical in itself. Shun citing references at this point.

An abstract is a brief distinct paragraph summary of finished work or work in development. In a minute or less a reviewer can be taught the foundation behind the study, common approach to the problem, relevant results, and significant conclusions or new questions.

Write your summary when your paper is completed because how can you write the summary of anything which is not yet written? Wealth of terminology is very essential in abstract. Yet, use comprehensive sentences and do not let go readability for briefness. You can maintain it succinct by phrasing sentences so that they provide more than lone rationale. The author can at this moment go straight to shortening the outcome. Sum up the study, with the subsequent elements in any summary. Try to maintain the initial two items to no more than one ruling each.

- Reason of the study - theory, overall issue, purpose
- Fundamental goal
- To the point depiction of the research
- Consequences, including definite statistics - if the consequences are quantitative in nature, account quantitative data; results of any numerical analysis should be reported
- Significant conclusions or questions that track from the research(es)

Approach:

- Single section, and succinct
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- Center on shortening results - bound background information to a verdict or two, if completely necessary
- What you account in an conceptual must be regular with what you reported in the manuscript
- Exact spelling, clearness of sentences and phrases, and appropriate reporting of quantities (proper units, important statistics) are just as significant in an abstract as they are anywhere else

Introduction:

The **Introduction** should "introduce" the manuscript. The reviewer should be presented with sufficient background information to be capable to comprehend and calculate the purpose of your study without having to submit to other works. The basis for the study should be offered. Give most important references but shun difficult to make a comprehensive appraisal of the topic. In the introduction, describe the problem visibly. If the problem is not acknowledged in a logical, reasonable way, the reviewer will have no attention in your result. Speak in common terms about techniques used to explain the problem, if needed, but do not present any particulars about the protocols here. Following approach can create a valuable beginning:

- Explain the value (significance) of the study
- Shield the model - why did you employ this particular system or method? What is its compensation? You strength remark on its appropriateness from a abstract point of vision as well as point out sensible reasons for using it.
- Present a justification. Status your particular theory (es) or aim(s), and describe the logic that led you to choose them.
- Very for a short time explain the tentative propose and how it skilled the declared objectives.

Approach:

- Use past tense except for when referring to recognized facts. After all, the manuscript will be submitted after the entire job is done.
- Sort out your thoughts; manufacture one key point with every section. If you make the four points listed above, you will need a least of four paragraphs.

- Present surroundings information only as desirable in order hold up a situation. The reviewer does not desire to read the whole thing you know about a topic.
- Shape the theory/purpose specifically - do not take a broad view.
- As always, give awareness to spelling, simplicity and correctness of sentences and phrases.

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This part is supposed to be the easiest to carve if you have good skills. A sound written Procedures segment allows a capable scientist to replacement your results. Present precise information about your supplies. The suppliers and clarity of reagents can be helpful bits of information. Present methods in sequential order but linked methodologies can be grouped as a segment. Be concise when relating the protocols. Attempt for the least amount of information that would permit another capable scientist to spare your outcome but be cautious that vital information is integrated. The use of subheadings is suggested and ought to be synchronized with the results section. When a technique is used that has been well described in another object, mention the specific item describing a way but draw the basic principle while stating the situation. The purpose is to text all particular resources and broad procedures, so that another person may use some or all of the methods in one more study or referee the scientific value of your work. It is not to be a step by step report of the whole thing you did, nor is a methods section a set of orders.

Materials:

- Explain materials individually only if the study is so complex that it saves liberty this way.
- Embrace particular materials, and any tools or provisions that are not frequently found in laboratories.
- Do not take in frequently found.
- If use of a definite type of tools.
- Materials may be reported in a part section or else they may be recognized along with your measures.

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- Report the method (not particulars of each process that engaged the same methodology)
- Describe the method entirely
- To be succinct, present methods under headings dedicated to specific dealings or groups of measures
- Simplify - details how procedures were completed not how they were exclusively performed on a particular day.
- If well known procedures were used, account the procedure by name, possibly with reference, and that's all.

Approach:

- It is embarrassed or not possible to use vigorous voice when documenting methods with no using first person, which would focus the reviewer's interest on the researcher rather than the job. As a result when script up the methods most authors use third person passive voice.
- Use standard style in this and in every other part of the paper - avoid familiar lists, and use full sentences.

What to keep away from

- Resources and methods are not a set of information.
- Skip all descriptive information and surroundings - save it for the argument.
- Leave out information that is immaterial to a third party.

Results:

The principle of a results segment is to present and demonstrate your conclusion. Create this part a entirely objective details of the outcome, and save all understanding for the discussion.

The page length of this segment is set by the sum and types of data to be reported. Carry on to be to the point, by means of statistics and tables, if suitable, to present consequences most efficiently.You must obviously differentiate material that would usually be incorporated in a study editorial from any unprocessed data or additional appendix matter that would not be available. In fact, such matter should not be submitted at all except requested by the instructor.

Content

- Sum up your conclusion in text and demonstrate them, if suitable, with figures and tables.
- In manuscript, explain each of your consequences, point the reader to remarks that are most appropriate.
- Present a background, such as by describing the question that was addressed by creation an exacting study.
- Explain results of control experiments and comprise remarks that are not accessible in a prescribed figure or table, if appropriate.
- Examine your data, then prepare the analyzed (transformed) data in the form of a figure (graph), table, or in manuscript form.

What to stay away from

- Do not discuss or infer your outcome, report surroundings information, or try to explain anything.
- Not at all, take in raw data or intermediate calculations in a research manuscript.
- Do not present the similar data more than once.
- Manuscript should complement any figures or tables, not duplicate the identical information.
- Never confuse figures with tables - there is a difference.

Approach

- As forever, use past tense when you submit to your results, and put the whole thing in a reasonable order.
- Put figures and tables, appropriately numbered, in order at the end of the report
- If you desire, you may place your figures and tables properly within the text of your results part.

Figures and tables

- If you put figures and tables at the end of the details, make certain that they are visibly distinguished from any attach appendix materials, such as raw facts
- Despite of position, each figure must be numbered one after the other and complete with subtitle
- In spite of position, each table must be titled, numbered one after the other and complete with heading
- All figure and table must be adequately complete that it could situate on its own, divide from text

Discussion:

The Discussion is expected the trickiest segment to write and describe. A lot of papers submitted for journal are discarded based on problems with the Discussion. There is no head of state for how long a argument should be. Position your understanding of the outcome visibly to lead the reviewer through your conclusions, and then finish the paper with a summing up of the implication of the study. The purpose here is to offer an understanding of your results and hold up for all of your conclusions, using facts from your research and generally accepted information, if suitable. The implication of result should be visibly described. Infer your data in the conversation in suitable depth. This means that when you clarify an observable fact you must explain mechanisms that may account for the observation. If your results vary from your prospect, make clear why that may have happened. If your results agree, then explain the theory that the proof supported. It is never suitable to just state that the data approved with prospect, and let it drop at that.

- Make a decision if each premise is supported, discarded, or if you cannot make a conclusion with assurance. Do not just dismiss a study or part of a study as "uncertain."
- Research papers are not acknowledged if the work is imperfect. Draw what conclusions you can based upon the results that you have, and take care of the study as a finished work
- You may propose future guidelines, such as how the experiment might be personalized to accomplish a new idea.
- Give details all of your remarks as much as possible, focus on mechanisms.
- Make a decision if the tentative design sufficiently addressed the theory, and whether or not it was correctly restricted.
- Try to present substitute explanations if sensible alternatives be present.
- One research will not counter an overall question, so maintain the large picture in mind, where do you go next? The best studies unlock new avenues of study. What questions remain?
- Recommendations for detailed papers will offer supplementary suggestions.

Approach:

- When you refer to information, differentiate data generated by your own studies from available information
- Submit to work done by specific persons (including you) in past tense.
- Submit to generally acknowledged facts and main beliefs in present tense.

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Introduction	Containing all background details with clear goal and appropriate details, flow specification, no grammar and spelling mistake, well organized sentence and paragraph, reference cited	Unclear and confusing data, appropriate format, grammar and spelling errors with unorganized matter	Out of place depth and content, hazy format
Methods and Procedures	Clear and to the point with well arranged paragraph, precision and accuracy of facts and figures, well organized subheads	Difficult to comprehend with embarrassed text, too much explanation but completed	Incorrect and unorganized structure with hazy meaning
Result	Well organized, Clear and specific, Correct units with precision, correct data, well structuring of paragraph, no grammar and spelling mistake	Complete and embarrassed text, difficult to comprehend	Irregular format with wrong facts and figures
Discussion	Well organized, meaningful specification, sound conclusion, logical and concise explanation, highly structured paragraph reference cited	Wordy, unclear conclusion, spurious	Conclusion is not cited, unorganized, difficult to comprehend
References	Complete and correct format, well organized	Beside the point, Incomplete	Wrong format and structuring

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