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Research paper

Proximate, GC-FID, and micronutrient analysis of extracts of *azadirachta indica*

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Abstract

Different extraction media applied on the pulverized leaves of Neem plant (Azadirachta indica) were analyzed for its proximate, phytochemical, and micronutrient compositions, predominantly using the gas chromatographic technique. The results showed that the ethanol extract contained the highest amounts of carbohydrates and fibre. No significant difference ($p>0.05$) was recorded for the protein and ash content of both ethanol and methanol extracts, while the moisture and fat contents occurred highest in aqueous and methanol extracts respectively. The phytochemical screening revealed the absence of glycosides in all the extracts while steroids were found only in methanol and ethanol extracts. The aqueous extracts contained greater amounts of epicate chin (13.42%), lunamarine (5.81%), tannin (19.18%) and phytates (0.27%), but lacked anthocyanin, phenol and kaempferol, while rutin (77.54%), ribalinidine (2.06%), oxalate (1.23%), anthocyanin (1.16%), and sparteine (0.05%) occurred highest in the ethanol extract. Only sparteine was lacking in the methanol extract. No significant difference was recorded between the aqueous and ethanol extracts for the vitamin A, D, C, and B_2 contents, while except for vitamin B_3 (0.22mg/100g) and vitamin K (0.08mg/100g), 08mg/100g), the methanol extracts contained the least amounts of the vitamins evaluated. Manganese, zinc, copper, calcium and lead contents of the methanol extract were significantly higher than those of the other extracts while the aqueous extract contained the highest amount of sodium. This study has provided the scientific backing for the application of a specific extraction medium during the exploitation of distinct phytochemicals, while water, ethanol, and methanol should be the preferred extraction media for vitamins, proximate and dietary mineral contents respectively.

Keywords: *Proximate; Vitamins; Minerals; Phytochemicals; Neem Plant*

1. Introduction

Herbal medicine is regarded as one of the foremost medical practices believed to have been central to ushering in the present modern pharmaceutical trade. Plants with well known medical uses are referred to as medicinal plants, and these plants are found growing in different localities over the world. With such a long history of working with medicinal plants, most chemists have vested interests in plants with paucity of information in order to investigate their active components and their corresponding mechanisms of action. In most cases, the target is to produce a synthetic version of the compound reproducible in the laboratory and utilizable by pharmaceutical companies. Medicinal plants are used by over 80% of the rural communities all over the world for the provision of primary health care services (Prajapati and Prajapati, 2002; Latif *et al*., 2003; Shinwari *et al*., 2006) and besides the presence of pharmacologically active components, each plant posses a unique nutrient content. The nutrients also contribute towards the satisfaction of energy requirements for various metabolic processes (Hoffman *et al*., 1998; Matthews *et al*., 1999; Dingman *et al*., 2002). Further, clinicians and medical practitioners are faced with the challenge of minimizing the numerous cases associated with drug resistance, and the screening of medicinal plants for their active components has received enormous attention as a suitable alternative. Natarajan *et al*., (2003) reported that manipulations of these compounds enable the provision of improved drugs used for the treatment of various diseases. The Azadirachta indica plant (known as dogonyaro in Nigeria) taxonomy is of the Meliaceae family (Callahan, 2010; Bharti *et al*., 2012). It is a tree that blossoms during the dry season with height of about 20-23m (Girish and Neem, 2008). It is a decidous plant that sheds its leaves during the harmattan periods in Nigeria. The neem branches spread widely revealing the fairly roundish dense crown (Krishan et. al., 2011). The neem tree (*Azadirachta indica*) also known as Nim tree and India lilac is very similar in appearance to its relative known as China-berry (*Mellia azedarac*h) which is a mahogany family of Meliaceae (Krishan *et al*., 2011) of Azadirachta indica species. Neem is widely used for its various therapeutic effects. According to the reports of Sithisarn *et al*., (2005) and Ghimeray *et al*., (2009) the leaves of neem have proven antioxidant properties, while Kumar *et al*., (2010) have shown the antimutagenic effects of neem against 7,12-dimethylbenz(a)anthracene. Further, other notable medicinal properties of neem include hepatoprotective effect (Bhanwra, 2000; Baligar *et al*., 2014), anti-inflammatory (Kaur *et al*., 2004; Mosaddek and Rashid, 2008), antinephrotoxic effect (Abdel Moneim *et al*., 2014), neuroprotective effects (Abdel Moneim, 2014), and growth promoting and immunomodulatory effects (Durrani *et al*., 2008). Notwithstanding these reported medicinal properties to the neem plant, the utilization of different extraction solvents has led to widely differing outcomes. This might be related to the ability of these solvents to extract different phytoconstituents both in amount of and properties and amount. It is on this note that this study compared the

nutrient contents of Azadirachta indica, on application of various extraction mediums.

2. Materials and methods

2.1. Sample collection and preparation

Fresh leaves of Azadirachta indica were obtained from bushes in University of Port Harcourt. The leaves were thoroughly washed with water to remove debris and air dried. The samples were ground using a mechanical grinder and soaked in water, ethanol, and methanol prior to analysis.

2.2. Determination of proximate composition

Proximate analyses of the samples for carbohydrate, crude fat, ash, crude protein, fiber and moisture contents were carried out according to standard methods of AOAC (1990).

2.3. Phytochemical screening and phytochemical determination using GC-FID method

The methods of Sofowora, (1980) and Harborne (1973) were applied for the qualitative detection of phytochemicals in the samples. Quantitative phytochemical analysis of the oil samples was carried out using an auto system buck 530 chromatographer in gas phase equipped with an on-column automatic injector, flame ionization detector, and with Hp88 capillary column (100m x 0.25mm). Chromatographic conditions were; injector temperature 220^{o}C, detector temperature 250^{o}C, oven temperature to 180^{o}C, injection volume 1ml sample, hydrogen was used as a carrier gas (24 pound per square inch (PSI)). The concentration of each active component was determined based on the ratio between the area and mass of internal standard and area under the peaks of the phytochemicals identified.

2.4. Determination of vitamin composition

The vitamin content was analyzed by a modification of AOAC, (2006). The sample was subjected to the laboratory atmospheric condition on the bench after removing the samples from the storage chamber at 4^{0}C. The sample was pressed and completely homogenized in the mortar carefully with pestle to avoid forming balls. The homogenized sample (0.10g) was weighed into a beaker after extraction; the extract was concentrated to 1. 0ml and for analysis, using a HP 6890 Gas chromatographic apparatus fitted with pulse flame photometric detector (PFPD) using Nitrogen as carrier gas. Split ratio 20:1 with flow rate of 1.0ml/min, 0ml/minimum, inlet temperature 250^{0}C, and column type HP-5 with 30m x .25mm x .25 µm column dimensions. Oven temperature: initial temperature @ 50^{0}C for 2 min, detector temperature maintained 3200C; pressure 20psi and compressed air pressure 30psi.

2.5. Mineral content, mineral ratio and phytate determination

Wet digestion of samples (5ml) using a mixture of concentrated HNO_3 and 60% (v/v) $HClO_4$ was carried out according to the method of AOAC (1990) where the organic matter in the sample was digested and afterwards diluted to a final volume of 25 ml with deionized distilled water. The levels of Na, K, Ca, Fe, Mg, P, Mn, Cu, and Zn in the samples were thus evaluated using an atomic absorption spectrophotometer (Buck Scientific model 210 VGP) and flame photometer (Jenway model). The sulphate contents of the food samples were determined turbidimetrically according to AOAC (1984). The chloride level was determined titrimetrically using the method of AOAC, (1984).

3. Results and discussion

The proximate composition of various extracts of Azadirachta indica is shown in Fig. 1 below.

The moisture content of the aqueous extract was significantly ($p<0.05$) higher than that for the methanol, and ethanol extract. The moisture contents of the three extracts were all higher than the reports for neem plant shown by Kashif and Ullah, (2013) and Madaki *et al*., (2016). The ash contents of all the extracts were comparable while the ethanol extracts produced the highest amount of fibre. The ash and fibre content of this plant under study were lower than those for *Sida acuta* (Enin *et al*., 2014), *Vernonia amygdalina, and Gongronema latifolium* (Atangwho *et al*., 2009). The implication with such low fibre content is that the leaves of Neem plant might not be suitable to relieve constipation. The ash content may imply poor mineral composition of this plant under study. The result of Fig. 1 showed methanol as the most suitable solvent in the extraction of fats and proteins. The fat and protein content of the methanol extract was in agreement with the reports of Atangwho *et al*., (2009) for Neem plant, and higher than those for *Occimum tenniflorumi* (Kashif and Ullah) and *Withania somnifera* leaves (Ishfaq and Farrukh, 2015). No significant difference ($p>0.05$) was observed for the carbohydrate contents extracted with either water or ethanol. The carbohydrate content of the ethanol extracts was comparable to that of *Emilia coccinea* but lower than that of *Anthecleista djalonensis, Spigella anthelmia* and *Ageratum conyzoides* (Nduche *et al*., 2015).

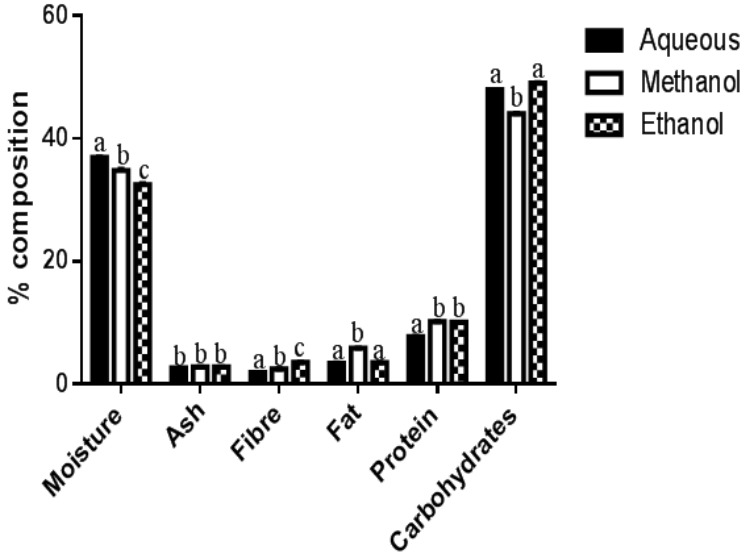

Fig. 1: Proximate Composition of Different Extracts of Plant Bars with Similar Superscript Letter (A, B, C) are Not Significantly Different (P > 0. 05).

Table 1: Phytochemical Screening of Various Extracts of Neem Plant

Phytochemicals	Aqueous extract	Methanol extract	Ethanol extract
Alkaloids	+	+	+
Carbohydrates	+	+	+
Reducing Sugars	+	+	+
Flavonoids	+	+	+
Tannins	+	+	+
Phenols	+	+	+
Triterpenoids	-	+	+
Saponins	+	+	+
Oxalate	+	+	+
Glycosides	_	_	-
Steroids	_	+	+

+ means present, - means absent.

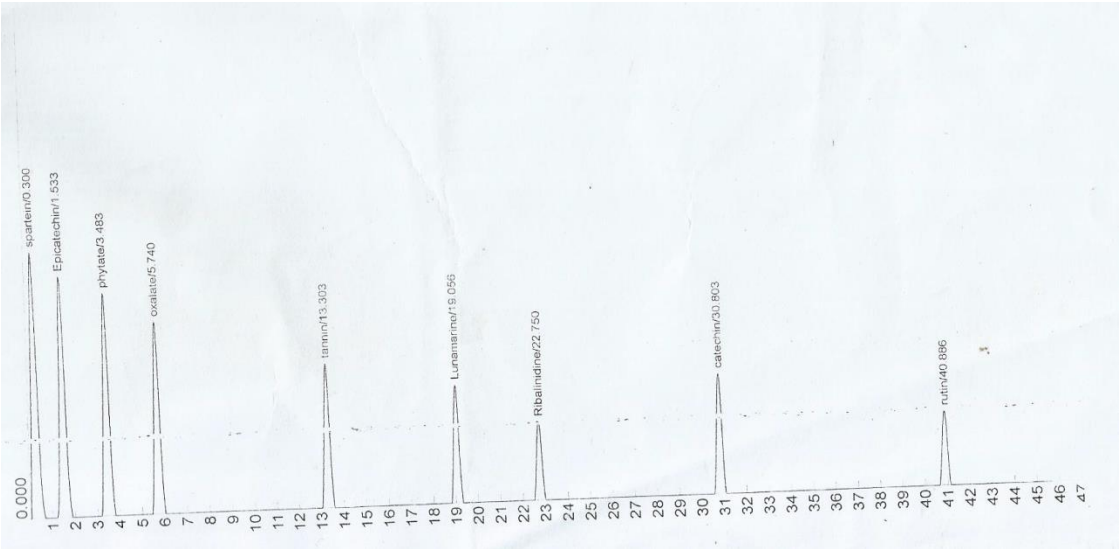

Fig. 2: Chromatogram for Phytochemical Analysis of Neem Plant Aqueous Extract.

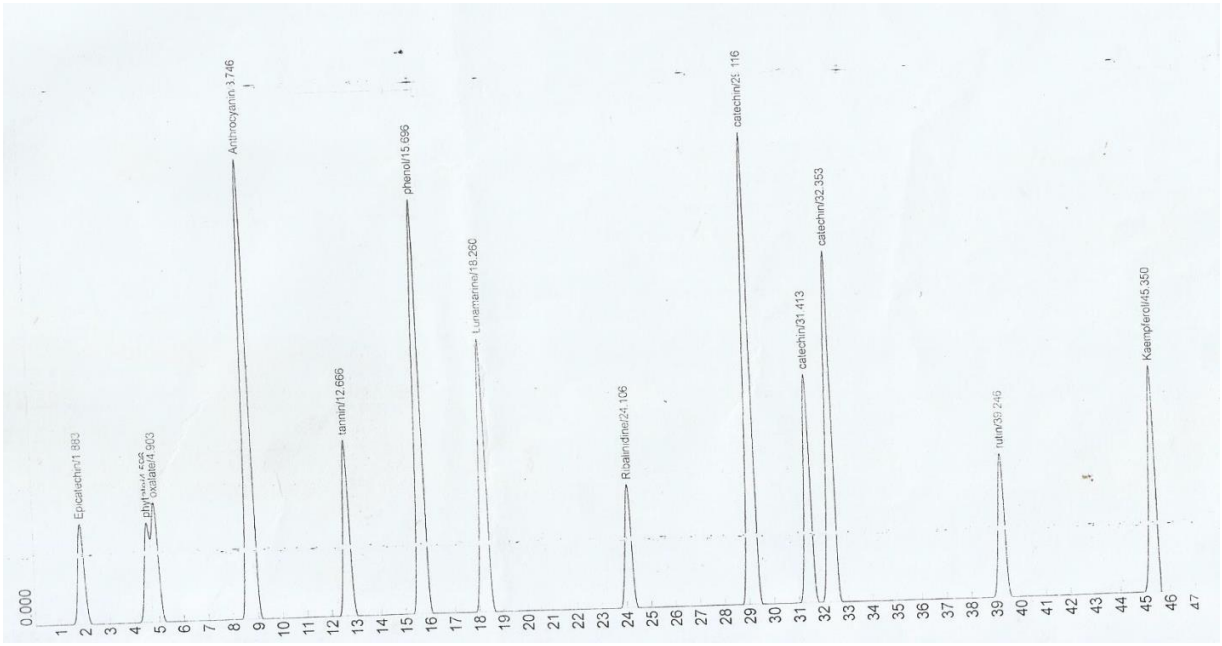

Fig. 3: Chromatogram for Phytochemical Analysis of Neem Plant Methanol Extract.

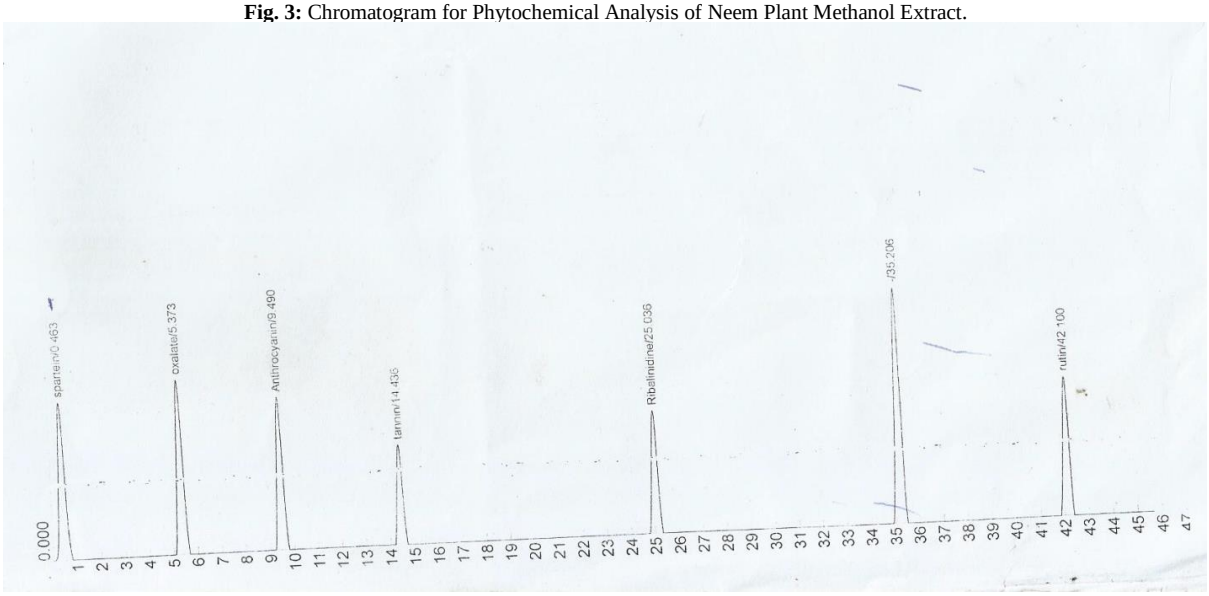

Fig. 4: Chromatogram for Phytochemical Analysis of Neem Plant Ethanol Extract.

Table 2: Phytochemical Analysis of Various Extracts of Neem Plant

Phytochemical	Aqueous extract (%)	Methanol extract (%)	Ethanol extract (%)
Sparteine	0.03	ND	0.05
Epicatechin	13.42	11.60	ND
Oxalate	1.03	0.18	1.23
Anthocyanin	ND	0.76	1.16
Ribalinidine	1.04	1.05	2.06
Catechin	25.85	27.34	ND
Phenol	ND	4.3	ND
Rutin	34.40	26.40	77.54
Kaempferol	ND	8.27	ND
Lunamarin	5.81	3.78	ND
Tannin	19.18	16.3	17.76
Phytate	0.27	0.02	0.20

ND means not detected

The result for the qualitative phytochemical composition of Azadirachta indica is shown in Table 1. The results confirm the presence of alkaloids, carbohydrates, reducing sugars, flavonoids, tannins, phenols, and saponins. A similar result was reported by Njoku and Obi, (2009) for *C. papaya* and *Adenia cissampeloides*. Alkaloids are known to confer several essential physiological effects on humans and animals. The very bitter taste of this plant under study may be attributed to its tannin content. The presence of flavonoids and phenos indicate a possible antioxidant property of *A. indica*. Only ethanol and methanol extracts showed the presence of triterpenoids and steroids. Siddiqui *et al*., (2000) have isolated nimocinol, a triterpenoid from neem plant, with possible insecticidal activity against *Aedes aegypti*. The presence of steroids in neem plant has also been confirmed in similar studies (Ejoba, 2012). Ejoba, (2012) reported the absence of saponins from aqueous extract of neem plant, which is not in line with the findings of this present study. The presence of saponins indicates possible usage of these plant extracts as a cleaning agent. Oxalate

was not confirmed in only the ethanol extracts of this plant while no glycoside activity was recorded in any of the extracts. Fig. 2-5 shows the chromatograms for the phytochemical analysis of various extracts of *A. indica*. Table 2 shows the phytochemical analysis of aqueous, methanol, and ethanol extracts of *A. indica*. Among the three extracts used, methanol proved the least effective in extraction of the alkaloid; sparteine, lunamarine, and ribalinidine. The forkloric claims of this plant usage in medicine for the stimulation of the cardiac and uterine muscles in childbirth might be related to these alkaloid activities. The phenol epicatechin and catechin were highest in the aqueous and methanol extract respectively, while total phenol was only found in the methanol extract. The phenols' catechin and epicatechin have been found to improve various heart conditions most especially normalizing blood pressure. This might be the reason behind the prescription of neem plant by herbalists, for antihypertension therapy. The oxalate levels of both ethanol and aqueous extracts were higher than the methanol extracts. Osuntokun and Oluwafoise, (2015) reported higher oxalate contents for *Pseudocedrela kotschyi* when compared to those presented for the various extracts in this study while the report of Nwachoko and Jack, (2015) for *Tetracarpidium conophorum* was lower than that for *A. indica* in this study. The low oxalate composition indicates minimal or no digestive difficulties that could result from the consumption of the extracts of *A. indica*. The results for the flavonoids; anthocyanin, rutin, and kaempferol, showed that ethanol proved most effective in the provision of the flavonoids with the exception of kaempferol undetected in both aqueous and ethanol extract. The high rutin content of this plant may be in support of the reports of Shitlania *et al*., (2016) that reported the ameliorative antimalarial effects of the combination of rutin. Also, the very high rutin content may be connected to the antidiabetic activity of neem plant with respect to the findings of Niture *et al*., (2014) on thea Anti-hyperglycemic activity of rutin in streptozotocin-induced diabetic rats. In addition, the rutin and the kaempferol content of *A. indica* provide the scientific backing behind it antimicrobial properties (Reddy *et al*., 2013). The aqueous extract provided the highest antinutrients (phytates and tannins). *A. indica* leave is a very bitter tasting plant. This astringency could be as a result of tannin content. None of the medicinal plant leaves; *Bauhinia cheilantha, Capparis jacobinae Moric, Nicotiana glauca, Lantana camara*, and *Croton rhamnifolius* examined by Clarissa *et al*., (2012) contained any comparable tannin content. The phytate compositions of the aqueous and ethanol extract of *A. indica* in this study were comparable to those for *Vernonia amygdalina, Gnetum Africanum, Piper guinensis*, and *Pterocarpus santolinoides* (Odoemelam *et al*., 2015). The formation of stable complexes of divalent metals by phytates from phytate-rich foods, might not be an obtainable case with the various extracts of *A. indica* in this study, having shown low and comparable phytate compositions similar to popularly consumed vegetables.

Table 3: Vitamin Contents of Various Extracts of Neem Plant

Vitamins (mg/100g)	Aqueous	Methanol	Ethanol
Vitamin A	0.34 ± 0.01^{a}	0.12 ± 0.01^{b}	0.29 ± 0.04^{a}
Vitamin D	0.75 ± 0.14^{a}	0.77 ± 0.04^{a}	0.69 ± 0.11^{a}
Vitamin E	2.95 ± 0.08^{a}	1.09 ± 0.06^{b}	2.60 ± 0.07^{c}
Vitamin K	ND	0.08±0.01	ND
Vitamin C	36.86 ± 0.30^{a}	22.80 ± 0.43^{b}	35.71 ± 0.51^{a}
Vitamin B_1	1.55 ± 0.20^{a}	0.89 ± 0.14^{b}	0.83 ± 0.18^{b}
Vitamin B_2	0.15 ± 0.04^{a}	0.10 ± 0.02^{a}	0.13 ± 0.08^{a}
Vitamin B_3	0.14 ± 0.04^{a}	0.22 ± 0.02^{b}	0.09 ± 0.03^{c}

Values are means ± S.D of triplicate determinations. Values bearing dissimilar superscript letter (a, b, c) are significantly different ($p < 0.05$).

The vitamin compositions of various extracts of *A. indica* are shown in Table 3. The vitamin a content of the methanol extracts (0.12 mg/100g) was the least in occurrence, while no significant change was recorded between the vitamin A content obtained from both the aqueous (0.34 ± 0.01) and ethanol extract (0.29 ± 0.04). The level found in this study for vitamin A content was higher than that of Acalypha wilkesiana (Ikewuchi and Ikewuchi, 2009). No significant difference was recorded among the vitamin D contents of the three extracts, while the aqueous extract provided the highest content of vitamin E among the three extracts. Vitamin K was undetected in both aqueous and methanol extract of *A. indica* in this study. The levels of these fat-soluble vitamins in all the extracts shown in Table 3, were higher than those from the reports of Ikewuchi and Ikewuchi, (2009) for *Acalypha wilkesiana, Chromolaena odorata*, and *Tridax procumbens*. With these levels found in this study, in comparison to the RDA for the vitamins, the neem plant is an inadequate source for vitamin A and E, while the vitamin D and K levels at 100mg was found sufficient. Vitamin A plays an important role in the maintenance of good vision, while vitamin D enhances the calcium homeostasis as well as promotes the uptake of phosphorus and calcium (Bertone-Johnson *et al*., 2005). Vitamin E is an important antioxidant that helps to relieve the body of oxidative stress while vitamin K is need for the activation of the several vitamin K-dependent clotting factors through its calcium binding potentials. Both the aqueous and ethanol extracts provided the highest vitamin C content (36.86 mg/100g and 35.71 mg/100g respectively). The vitamin C contents of the various extracts of neem plant in this study, was higher than those for the vegetables; *Portulaca oleracea Allium sativum*, and *Hibiscus esculentus* (Bangash *et al*., 2011) while *Spinacea oleracea* and *Momordica charantia* (Bangash *et al*., 2011) provided better sources of vitamin C, than the neem plant in this present study. Vitamin C is a well-known antioxidant, and as plays a role during the synthesis of derivatives of folic acid, essential for DNA synthesis (Chatterjea and Shinde, 1998). The thiamine (vitamin B_1) content of the aqueous extract of neem plant (1.55 mg/100g) as shown in Table 3 was significantly ($p<0.05$) higher than those for methanol (0.89 mg/100g) and ethanol extract (0.83 mg/100g). These values were higher than the reports of Okwu (2005) for *Aframomum melegueta* and *Garcinia kola*. Thiamine is involved in muscle function and nervous system, movement of electrolytes, and the digestive system. Further, no significant difference was recorded for the vitamin B_2 composition among the three extracts studied, while the usage of methanol for extraction proved most yielding for niacin (vitamin B_3) extraction. The riboflavin levels recorded in this study were lower than those for *Ranunculus arvensis, Equisetum ravens, Carathamus lanatus* and *Fagonia critica* (Hussain *et al*., 2011). The niacin content of the methanol extracts of neem plant in this study was comparable to that of garlic (*Allium sativum*), mustard (*Brassica campestris*) and bath sponge (*Luffa acutangula*), but lower than that of spinach (*Spinacea oleracea*) and egg plant (*Solanum melongena*) as reported by (Bangash *et al*., 2011). Riboflavin functions as a coenzyme and is required for numerous physiological functions such as cellular respiration Beta oxidation, vision, etc., and while niacin is useful for the treatment of pellagra and hypercholesterolemia.

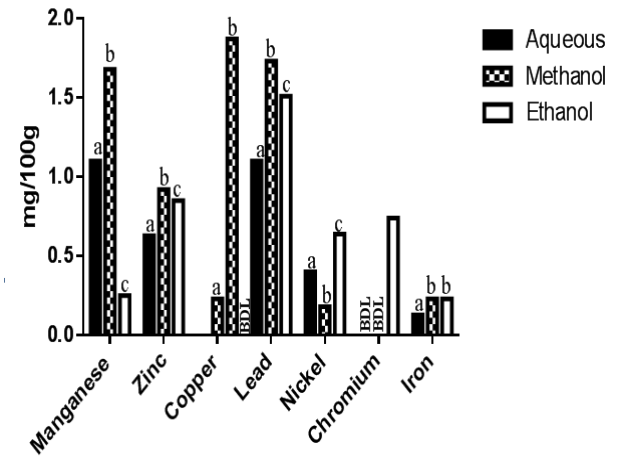

Fig. 5: Micro Mimeral Content of Various of Neem Plant BDL Means below Detection Levels. Bars with Similar Superscript Letter (A, B, C) are Not Significantly Different (P > 0. 05).

The micro mineral composition of various extracts of neem plant was shown in Fig. 5. The methanol extracts produced the significantly highest amount of manganese, zinc, copper and lead. The manganese and zinc contents of all the extracts of neem plant evaluated in this study were lower than those for *Pseudocedrela kotschyi, Anogeissus leiocarpus, Terminalia glaucescens* and *Zanthoxylun Lessmamul* (Osuntokun and Oluwafoise, 2015) while the copper and lead contents presented in this study for the extracts were higher than those for *Carica papaya, Nicotiana tabacum, Morinda lucida* and *Piper guineense* (Osuntoken, 2015). Zinc and manganese are both essential constituents of many enzymes and are required for their various activities while copper is required during the onset of haemopoiesis. The result of this study for lead suggests possible toxic effects that could arise from excessive consumption of neem plant. Further, ethanol provided the highest amount of nickel and chromium, though, chromium was undetected in both aqueous and methanol extract of the neem plant (Fig. 5). The result further indicated that methanol and ethanol provided a comparable amount of iron. Chromium was found below detection levels in the aqueous and methanol extracts whereas the chromium levels of the ethanol extract of neem plant in this study was not in agreement with the reports of Atangwho *et al*., (2009), though higher than those for *V. amygdalina* and *G. latifolium* (Atangwho *et al*., 2009), while the nickel content was higher than that reported by Offor, (2014). The lead contents of the methanol and ethanol content presented in this study were comparable.

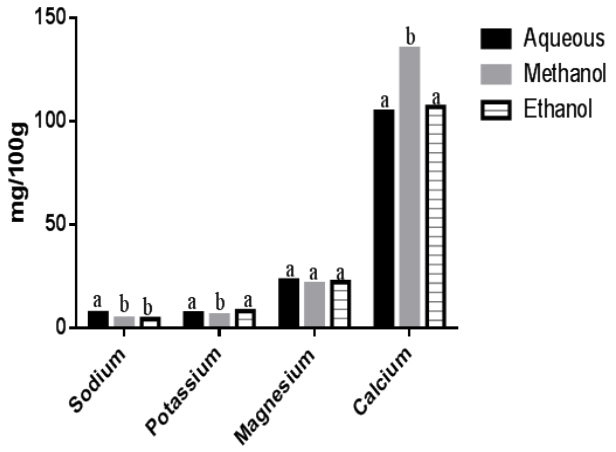

Fig. 6: Macro Mineral Content of Various Extracts of Neem Plant. Bars with Similar Superscript Letter (A, B, C) are Not Significantly Different (P > 0. 05).

The macro mineral content of various extracts of neem plant was presented in Fig. 6. No significant difference ($p > 0.05$) was recorded between the sodium content of the methanol and ethanol content of neem plant evaluated in this study. Ethanol produced the highest sodium content, and was higher than those for G. kola and *A. melegueta* (Okwu, 2005) but lower than that of *M. utilis* (Ujowundu *et al*., 2010). For the potassium content, both the ethanol and aqueous extract produced comparable amounts. From the reports of Enin *et al*., (2014) *S. acuta* produced higher potassium contents than that of neem plant in this study. *P. guineense, P. umbellatum,* and *S. striatinux* (Armand *et al*., 2013) similarly produced higher potassium contents than neem plant in this study. Further, the result indicated that all the extracts comparable amounts of magnesium. The medicinal plants; *Anogeissus leiocarpus, Terminalia glaucescens,* and *Zanthoxylun Lessmamul* according to Osuntokun and Oluwafoise, (2015) possessed similar amounts of magnesium compared to the neem plant in this study. In this study, the usage of methanol for extraction was most effective for the extraction of calcium (Fig. 6). No significant change was found for the calcium content of the aqueous and ethanol extract. The findings of this study for the calcium content were not in agreement with the reports of Sahito *et al*., (2003).

4. Conclusion

The current study of the analysis of different extracts of Azadirachta indica will enhance the applicability of this medicinal plant in various pharmaceutical and therapeutic processes. The findings of this study were indicative of general suitability of ethanol for extraction of proximate contents, aqueous extracts for vitamins, while methanol extracts provided the highest amounts of phytochemicals and minerals.

5. Conflict of interest

The authors declare no existing conflict of interest regarding this article.

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