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RESEARCH ARTICLE

Partial purification and antidiabetic effect of bioactive compounds isolated from medicinal plants

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ABSTRACT

Antidiabetic studies were conducted in human whole blood using different medicinal plant products (*Trigonella foenum graecum*, seeds; *Syzygium cumini*, seeds; *Salavadora persica*, leaves and *Terminalia chebula*, seeds). The objective of our study is to screen fractions of these four different medicinal plant products on diabetic human whole blood samples. For these studies, our group evaluated secondary metabolites through thin layer chromatography (TLC) and also determined its activity on diabetic human whole blood samples in order to determine total cellular content, free hemoglobin in the supernatant and also estimated its glucose content. The results of these studies claimed that all these fractions isolated from these medicinal plant productsshowed antidiabetic effect at lower doses because of decline in total cellular content, free hemoglobin in the supernatant and glucose content but maximum effect observed in case of fraction isolated from *Syzygium cumini* and *Salavadora persica*. Overall, this study claimed that all these plant fractions showed anti-diabetic activity.

Keywords: *Trigonella foenum graecum*; *Syzygium cumini*; *Salavadora persica*; *Terminalia chebula*; Antidiabetic.

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INTRODUCTION

Hyperglycemia, a biochemical hallmarkin the development of diabetes, assessed by glycated hemoglobin A1c or A1C levels to reduce the long term diabetes-related complications [1, 2]. In contrast undiagnosed or untreated hyperglycemia, leads to adverse consequences and have a longer length of stay.Mostly A1C therapy is advised when blood glucose level persistent within the range of >140-180 mg/dl as the blood glucose level >140 mg/dl is (7.8 mmol/L) considered as a hyperglycemia [3]. According to WHO report 2011, the cutoff value of HbA1c for diagnosis of diabetes is 6.5% (48 mmol/mol), while American Diabetes Association (ADA) advisedthe range 5.7-6.4% as a high risk range indicating the presence of intermediate hyperglycaemia [4]. Diagnosis using is more precise than that of FPG (fasting plasma glucose) and 2-h

OGTT (oral glucose tolerance test) diagnosis [5]. Available oral hypoglycemic agents such as biguanides and sulfonylurea have not yet gained much success in maintaining the proper glycemic condition maybe because of their side effects.Therefore, efforts are continuing with the development of oral antidiabetic agents without or less side effects[6]. Herbal drugs are preferred as alternative drug therapies for the conventional diabetic drugsas they are cost effective, tolerated by the patients, safer to use, etc. [7].

Medicinal plants have been used for centuries for the management and alleviation of Diabetes Mellitus (DM) and its complications. Much of the available literature in DM revealed that 1,200 medicinal plants with hypoglycemic activity [8]. Though the mechanism of these plants is not well documented, the majority of studies is being conducted to uncover the mechanism of action of these plants and their isolates.Hence, more attention is being paid to study the proper mechanism of these plant's actions along with their bioactive compounds responsible for these activities.*Galega officinalis L.* (Fabaceae) was the first medicinal plants described for its antidiabetic property [9]. In the present study, we have evaluated four plants in *vitro, viz. Fenugreek, Syzygium cumini, Salvadora persica* and *Terminalia chebula* for their antidiabetic activity on diabetic blood samples. All these plants are well documented for their pharmacological and medicinal properties, for example, aqueous extract of fenugreek when administered orally to the Male Wistar rats of 150 g in weight, shown improved carbendaziminduced testicular toxicity and thereby attributed to the antioxidant property [10]. Tannins and flavonoids, two bioactive compound identified in *Syzygium cumini*leaves aqueous extract, have shown anti-allergic and anti-edematogenic effect in Swiss mice. *Syzygium cumini* exerts these effects by inhibiting mast cell degranulation and histamine and serotonin effects on the cells [11]. Hydroalcoholic extract of *Syzygium cumini* leaves has also shown aneffective reduction in blood pressure and heart rate of spontaneously hypertensive Wistar rats by inhibiting arterial tone and extracellular calcium influx [12]. Tabatabaei and co-works have reported the time-dependent cell proliferative effect of aqueous extract of *Salvadora persica* on human dental pulp stem cells (hDPSCs) [13]. Ethanolic extract of *S. persica* is found to be effective in removing the smear layer from the coronal third of the canal wall [14]. In spite of the acting as a good anticaries agent [15], *Terminalia chebula* also showed anti-arthritic effect in six-week-old male DBA/1 mice by suppressing the expression of inflammatory mediators and preventing cartilage destruction and bone erosion. The bioactive compound chebulanin, isolated from fruit part is responsible to inhibit for the anti-arthritic collagen induced arthritis in DBA/1 mice [16]. The present study which described about antidiabetic potential of these fractions isolated from these medicinal plants i.e. *Trigonella foenum graecum, Syzygium cumini, Salavadora persica* and *Terminalia chebula.*

MATERIALS AND METHODS

Collection of plant material

Seeds of fenugreek and *T. chebula* were purchased and *S. cumini* seeds and *S. persica* leaves were collected from local region of Ahmednagar (MS), India. All these plant material were authenticated by the Department of Botany, Padmashri Vikhe Patil College, Pravaranagar (Loni), Tal. Rahata, District Ahmednagar, (MS), India.

Preparation of extracts

All plant samples (*Trigonella foenum graecum* seeds, *Syzygium cumini* seeds, *Salavadora persica* leaves and *Terminalia chebula* seeds) were ground into a fine powdered form and these samples were mixed with organic solvents i.e. chloroform for fenugreek, ethanol for *S. cumini*, water for *S. persica* and petroleum ether for *T. chebula.* All chemicals were purchased from Himedia. All the extracts were transfer toorbital shaker for 3 days at room temperature. The mixture wasthen filtered through Whatman No. 41 filter paper. All filtrate except aqueous extract, were concentrated using rotary evaporator. Filtered aqueous extracts of *Salavadora persica*, was frozen at -77°C and finally lyophilized at -46°C [17].

Thin Layer Chromatography

Thin Layer chromatography plates were prepared by applying 0.5 mm thick coat of silica gel (with distilled water in 1:2 ratio) on a microscope. Plates were activated at 110°C for 1h before use. 10 µl sample were applied to the plates and developed in chromatographic chamber with mobile phase appropriate to the extract as shown in Table 1. Plates were dried and spots were visualized with iodine chamber. Visualized spots were retrieved using an appropriate solvent [18].

Partial identification of nature related to bioactive compounds

Partial identification of nature of bioactive compounds isolated from all four plant extracts was carried out by using different chromogens giving colour reactions specific for alkaloids, flavonoids, saponins and tannins was added to extract and specific color pattern was

observed. For alkaloids, dragendorff‟s reagent was used and orange coloured demonstrated positive for alkaloids. For flavonoids, magnesium ribbon and concentrated hydrochloric acid were added and formation of red pink color was observed. For saponins, Anisaldehyde sulphuric acid reagent was added which gives pinkish violet color as an indication of a positive result. Screening for tannin was carried out by adding alcoholic $FeCl_3$. The greenish gray coloration confirmed the presence of tannins [19, 20].

Estimation of total cellular content and free hemoglobin content

Lysed diabetic human whole blood samples (n =5; 100 µl cells containing 10^5 cells/well) were collected (Mangal pathology lab, Baramati) and cultured in 24 well flat bottom tissue culture plate for 48 h incubation along with variable doses of these fractions isolated from *Trigonella foenum graecum* (fenugreek), *Syzygium cumini*, *Salavadora persica* and *Terminalia chebula*. In this study, huminsulin 50/50 used as standard and performed all these studies in two different set of experiments. In first set, centrifuge the samples at 15000 rpm after 48 h incubation and then collect the supernatant in order to determine the total cellular or protein content [21] using NanoDrop 1000 A280 module. In NanoDrop, Beer Lambert equation (A = E * b * c) is applied and used for all protein calculations to correlate absorbance with concentration. In second set of experiment, lysed diabetic human whole blood samples were treated with test material and incubate for 48 h and then washed with PBS pertaining to observe the free hemoglobin in the supernatant. Finally samples were analyzed through UV visible spectrophotometer at 570 nm [21].

Estimation of glucose content

Non infected lysed human whole blood samples were cultured for 48 h incubation along with variable doses of glucose content. After incubation, centrifuge the samples at 2500 rpm and estimate free glucose content in the supernatant. All these readings were determined and prepared standard curve pertaining to determine the glucose content in human diabetic blood samples which is determined through Nanodrop method.

Statistical analysis

The difference between control and treated group of medicinal plant products is controlled by one way ANOVA test (Bonferroni multiple comparison test). *P<0.05; **P<0.01 and ***P<0.001

RESULTS

Thin layer chromatography

Bioactive compounds isolated from these medicinal plant products using mobile phase chloroform and methanol along with retention factor (Rf values) and compared with reference values (Rv) which is mentioned in the literature as shown in Table 1. Overall, the results showed some similarity related to thin layer chromatography studies.

Estimation of total cellular content

The effect of these plant fractions on total cellular content as shown in Table 2. The results of these studies claimed that these plant fractions showed enhancement in total cellular content at higher doses as compared to control. The maximum effect of these plant fractions related to antidiabetic effect at lower doses in case of *Syzygium cumini* and *Salavadora persica* as compared to Huminsulin 50/50.

Estimation of free hemoglobin content

In addition, similar results also observed in free hemoglobin in the supernatant containing plant extracts (Fig. 1).

Table 1. Thin layer chromatography of medicinal plant products.

Plant Name	Bioactive compound	Mobile phase	Rf values	Reported values	References
Fenugreek	Saponin	C:M (15:1)	0.83, 0.95, 0.59	0.62, 0.75, 0.87, 0.90, 0.95, 0.98	Sharma et al. 2013 [21]
Syzygium cumini	Tannin	C:M (27:0.3)	0.37, 0.18, 0.13, 0.67, 0.83	0.26, 0.46, 0.66, 0.72.	Benmehdi et al. 2012 [22]
Salvadora persica	Flavonoid	C:M (18:2)	0.10, 0.21, 0.70, 0.97	0.18, 0.21, 0.68, 0.98	Iwashina et al. 2013 [23]
Terminalia chebula	Flavonoid	C:M (18:2)	0.94, 0.64, 0.30	0.37, 0.64	Victorio et al. 2009 [24]

C:M-chloroform: methanol.

Table 2. Effect of variable doses of fractions isolated from medicinal plant products on total cellular content.

Treatment	Doses (µg)	Total cellular content (mg/ml)	% Suppression/Stimulation
Control		0.278 ± 0.06	-
Trigonella foenum graecum (Fenugreek)	25	0.176 ± 0.02**	36.69 ↓
	50	0.242 ± 0.04	12.94 ↓
	100	0.322 ± 0.08	15.82 ↑
	200	0.388 ± 0.12	39.56 ↑
Control	-	0.278 ± 0.06	
Syzygium cumini	25	0.108 ± 0.02***	61.15 ↓
	50	0.186 ± 0.04**	33.09 ↓
	100	0.222 ± 0.04*	20.14 ↓
	200	0.314 ± 0.10	12.94 ↑
Control	-	0.278 ± 0.06	
Salvadora persica	25	0.148 ± 0.08**	46.76 ↓
	50	0.242 ± 0.10	12.94 ↓
	100	0.312 ± 0.14	12.23 ↑
	200	0.384 ± 0.12	38.12 ↑
Control	-	0.278 ± 0.06	
Terminalia chebula	25	0.198 ± 0.012*	28.77 ↓
	50	0.284 ± 0.06	2.15 ↑
	100	0.398 ± 0.14	43.16 ↑
	200	0.476 ± 0.24	71.22 ↑
Huminsulin 50/50	10	0.154± 0.02**	44.6 ↓

Lysed diabetic human whole blood (high glucose content) were cultured with variable doses of fractions isolated from medicinal plant products. Total cellular content was measured after high speed centrifugation and collect supernatant for estimation of total cellular content. Values are expressed as Mean ± S.E. The difference between control and variable doses of medicinal plant products is controlled by one way ANOVA test (Bonferroni multiple comparison test). *P<0.05; **P<0.01 and ***P<0.001.

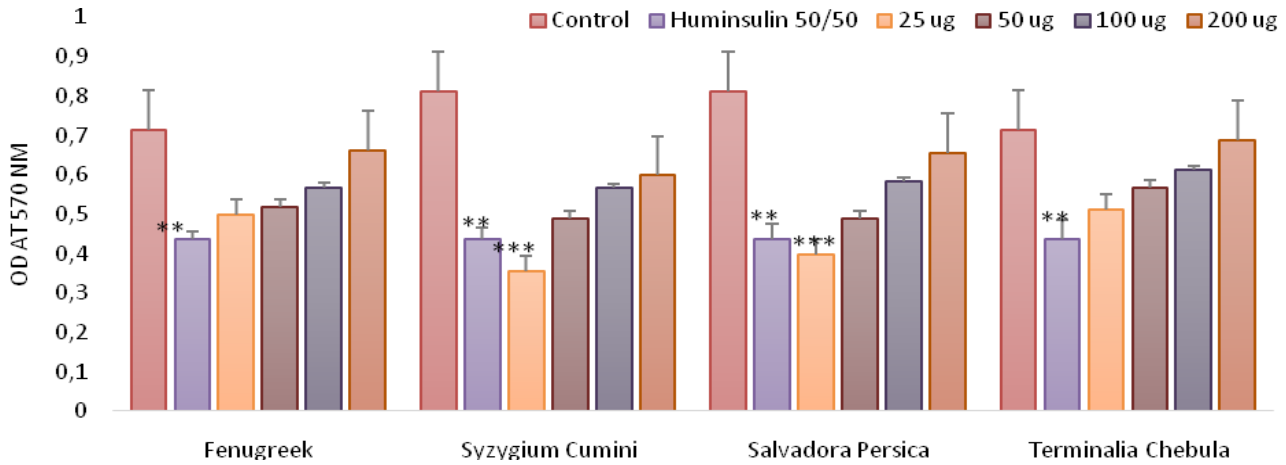

Fig.1. Estimation of free hemoglobin content in the supernatant of diabetic human whole blood samples containing plant extracts. Lysed diabetic human whole blood (high glucose content) were cultured with variable doses of medicinal plant products (as described in materials and methods section). Values are expressed as Mean ± S.E. The difference between control and variable doses of medicinal plant products is controlled by one way ANOVA test (Bonferroni multiple comparison test). *P<0.05; **P<0.01 and ***P<0.001.

Table 3. Effect of variable doses of fractions isolated from medicinal plant products on total glucose content in human diabetic blood samples.

Treatment	Doses (µg)	Total glucose content (mg/ml)	% Suppression/ Stimulation
Control		2.88 ± 0.16	-
Trigonella foenum graecum (Fenugreek)	25	1.88 ± 0.12**	34.72 ↓
	50	2.12 ± 0.08*	26.38 ↓
	100	2.94 ± 0.16	2.08 ↑
	200	3.48 ± 0.24	20.83 ↑
Control	-	2.88 ± 0.16	
Syzygium cumini	25	1.04 ± 0.004***	63.88 ↓
	50	1.76 ± 0.02**	38.88 ↓
	100	2.44 ± 0.78	15.27 ↓
	200	3.76 ± 0.68	30.55 ↑
Control	-	2.88 ± 0.16	
Salvadora persica	25	1.44 ± 0.04***	50.0 ↓
	50	1.98 ± 0.06**	31.25 ↓
	100	2.66 ± 0.04	7.63 ↓
	200	3.44 ± 0.18	19.44 ↑
Control	-	2.88 ± 0.16	
Terminalia chebula	25	2.08 ± 0.72**	27.77 ↓
	50	3.02 ± 0.98	4.86 ↑
	100	3.76 ± 0.74	30.55 ↑
	200	4.12 ± 0.54	43.05 ↑
Huminsulin 50/50	10	1.52 ± 0.44**	47.22 ↓

Non infected lysed human whole blood samples were cultured for 48 h incubation along with variable doses of glucose content. Values are expressed as Mean ± S.E. The difference between control and variable doses of medicinal plant products is controlled by one way ANOVA test (Bonferroni multiple comparison test). *P<0.05; **P<0.01 and ***P<0.001.

Overall, the results claimed that these plant extracts at lower doses showed antidiabetic effect because of decline in free hemoglobin in the supernatant. The maximum effect was observed in case of fractions isolated from *Syzygium cumini* and *Salavadora persica.* In addition, *Trigonella foenum graecum* (Fenugreek) and *Terminalia chebula* showed less anti-diabetic effect.

Estimation of glucose content

The effect of these test materials on glucose content in human whole blood samples as shown in Table 3. The results of these studies showed that these plant extracts especially *Syzygium cumini* and *Salavadora persica* showed declined in glucose content at lower doses as compared to Huminsulin 50/50.

DISCUSSION

Use of medicinal plant products for human health against various infectious diseases which is already mentioned in the literature. In other words, these medicinal plants plays a crucial role in the development of human health which is more affordable and easily accessible source of treatment. Recently there is increased interest in natural products among researchers for their utilization especially in case of prophylactic and therapeutic treatment which basically needs to isolate, purify and structurally characterize these active constituents as well as to study the biological activities of naturally occurring primary and secondary metabolites [25]. For these studies, thin layer chromatography is required to identify and separated the bioactive compounds. This technique is considered to be more versatile, inexpensive techniques

that quickly separate and isolate the biochemical compounds from mixture compounds. The method is also applicable in forensic science to identify drugs of abuse in body fluids [27]. In our study, we have applied this method to detect the biologically active phytochemicals. We have tried different concentration of the solvent system to achieve a better separation of the bioactive compounds. As shown in Table 1, our group separated bioactive compounds that are mentioned in the selected solvent systems. In our previous work, we had screened the different solvent extracts for their inhibition of alpha amylase activity [27-30]. The solvent that gave the best results (highest percent of alpha amylase inhibition) are further analyzed for their bioactive compound. Our values were well matched with the literature (Table 1) confirming that a petroleum ether extract of fenugreek contains saponins as a bioactive compound,the antidiabetic activity of *Syzygium cumini* is due to the presence of tannins while *Salvadora persica* and *Terminalia chebula* are rich in flavonoids that make them to become a better antidiabetic agent.

In this study, exposure of these fractions isolated from medicinal plant products caused a reduction in the blood glucoselevel and total cellular content at lower dosesin case of lysed diabetic human whole blood samples. The decreased glucose level in lysed diabetic human whole blood clearly showed the anti-hyperglycaemic effect of these fractions and seem to justify the claim of the traditional healers. Further studies are needed to confirm the hypoglycemic activity of this plant and to evaluate its potential in the treatment of diabetes. According to literature there are two factors those specially are associated with diabetic profile, low hemoglobin concentration in blood as well as high blood glucose concentration in the blood [31, 32]. Regulation of these component using herbal treatment is the goal of the study.Scientific validation of these fractions isolated from medicinal plant productswhich has proved its efficacy in order to reducing the sugar level which is confirmed and detected through free hemoglobin content and glucose estimation in lysed human diabetic whole blood samples. From these results related to its potential effectiveness against diabetes, it is assumed that these fractions isolated from medicinal plant products that played in the management of diabetes, which needs further exploration for necessary development of drugs and nutraceuticals from natural resource.

CONCLUSION

By analyzing the result we have concluded thatsecondary metabolites isolated from four plants *viz. Trigonella foenum graecum* (Fenugreek), *Syzygium cumini*, *Salavadora persica* and *Terminalia chebula* possess and showed potent antidiabetic activity. Further investigation is required to purify the exact bioactive compound which can beserved as a potent antidiabetic drug.

AUTHORS CONTRIBUTION

This work was carried out in collaboration between four authors. AG designed the study, wrote the protocol and interpreted the data where TB anchored the field study, gathered the initial data related to her PhD work under AG guidance and performed preliminary data analysis. AG, TB, BS and BKS managed the literature searches whereas AG and TB produced the initial draft. The final manuscript has been read and approved by all authors.

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RESEARCH ARTICLE

Evaluation of lactate dehydrogenase isoenzyme activity and biochemical profile in cerebrospinal fluid for different types of meningitis

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ABSTRACT

Meningitis is life-threatening condition and examination of the cerebrospinal fluid (CSF) may not give a precise diagnosis and prognosis of different types of meningitis. Bacterial meningitis is still a very common problem especially in many developing countries. The aims of study are to estimate the importance and significance of lactate dehydrogenase (LDH) isoenzymes, protein and sugar in CSF of different types of meningitis. A total of 160 cases, aged between 1 month and 60 years, including patients with bacterial meningitis (n=50), pyogenic meningitis (n=46), viral meningitis (n=24) and a control group (n=40), were analyzed on the basis of data from the initial clinical examinations. Significant increase in LDH level (P<0.001) were observed in the test group when compared to the control group. The LDH activity was significantly elevated in the CSF and serum ($p < 0.001$) in cases of pyogenic (PM) as well as tuberculous meningitis (TBM). Bacterial meningitis is more common than non bacterial meningitis. The enzymatic activity of LDH although significantly raised in PM compared to TBM but there was no cutoff level to differentiate them. The LDH level did rise quite significantly in pyogenic meningitis.

Keywords: Lactate dehydrogenase (LDH); Cerebrospinal fluid (CSF); Meningitis.

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INTRODUCTION

Lactate dehydrogenase (LDH) is zinc containing enzymes is present in most tissues and body fluids. [1]. Thereafter, many workers found significant elevations of CSF-LDH level in meningitis and other cerebrovascular disorders. Lactate dehydrogenase is intracellular enzyme, it is sensitive indicator of bacterial meningitis [2, 3]. LDH have four isoenzymes (LDH1, LDH2, LDH3 and LDH4), LDH1 preponderance, followed by LDH-2, LDH-3 and LDH-4. Different patterns are indicative of different diseases. For example, patients with bacterial meningitis show elevated levels of LDH-4 and LDH-5 [4]. The reports indicated that an increase in CSF level of lactate dehydrogenase might be of potential value in diagnosing bacterial meningitis when CSF findings of protein, sugar

and cells are non-specific. Bacterial meningitis is still a considerable cause of mortality and morbidity especially in children [5-7]. Although many studies have acknowledged the CSF in either diagnosis or prognosis of bacterial meningitis patients [8-10] recent studies however emphasize the fact that absence or low levels of CSF (especially after 12 hours manifestation of clinical symptoms) strongly rule out bacterial meningitis [11].

The aims of the study are to find out the role of LDH isoenzymes activity in CSF to differentiate among pyogenic, tuberculous and viral meningitis.

MATERIALS AND METHODS

Total 160 CSF samples were examined. Out of them 120 patients of all age groups and either sex of clinically suspected cases of meningitis were taken as test group. 40 control subjects of all age and either sex having no neurological, hepatic, muscular, and cardiac disorders were taken as control group. The study was approved by the Ethical Committee. Cerebrospinal fluids were collected by the lumbar puncture with all aseptic and antiseptic precautions were taken in a clean, dry and sterile vial, the amount of withdrawn CSF is not fixed, but usually in the range of 1-3 ml. CSF was centrifuged at 3000 rpm for 10 minutes and estimation of LDH, was done with clear supernatant parts of CSF. LDH was estimated by UV kinetic method (using SIGMA Kit) by semi-auto analyzer. All data are expressed as mean ± S.D. The data were also tested using student's t-tests; the significance level was set as $p < 0.05$.

RESULTS

In control group the mean ± S.D of CSF - LDH, protein and sugar levels were 31.0 ± 9.47, 29.90 ± 5.26 and 65.65 ± 5.48 respectively, which were within normal range.

The CSF total LDH and its isoenzymes LDH-1, LDH-2 and LDH-3 apparently originate from brain tissue, which contains the same isoenzymes (2, 5, 10). The CSF LDH isoenzyme patterns in the control group, LDH-1 was found to be the main fraction, followed by LDH-2 and then LDH-3; only small percentages of LDH-4 and trace percentages of LDH- 5 were detected (Fig. 1). By contrast, in patients with different types of meningitis, the LDH-1 fraction percentage was lower than that in the control group, whereas LDH-2 and LDH-3 were preponderant in almost all cases (Fig. 2). The CSF-LDH level in pyogenic meningitis is ranged from 181-333 IU/L with mean of 247.65 ± 37.58, which is statistically highly significant than control (P<0.0001). The protein level in pyogenic meningitis is ranged from 90-450 mg/100 ml with mean of 226.95 ± 138.47, which is statistically highly significant than control (P<0.0001). The sugar level in pyogenic meningitis is ranged from 10-36 mg/100 ml with mean of 23.87 ± 8.98, which is statistically highly significant than control (P<0.0001) (Table 2).

Table 1. Distribution of CSF - LDH level with other parameters in control group.

No. of cases (40)	Range	Mean	S.D.	SEM
LDH IU/L	10 – 44	31.0	9.47	1.50
Protein mg/100 ml	18 – 36	29.90	5.26	0.83
Sugar mg/100 ml	56 – 76	65.65	5.48	0.87

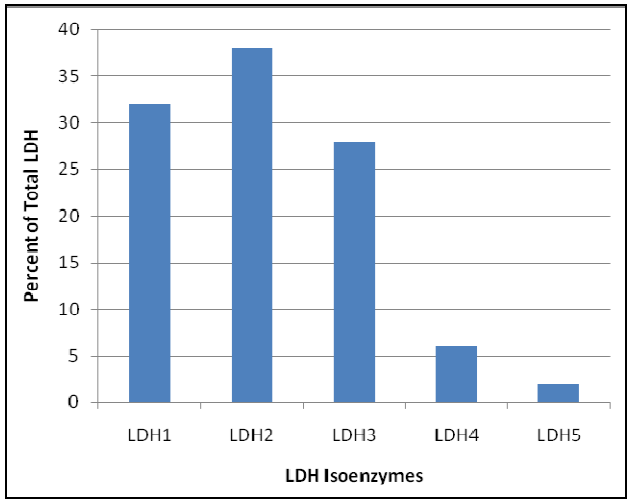

Figure 1. Normal lactic dehydrogenase (LDH) isoenzyme pattern.

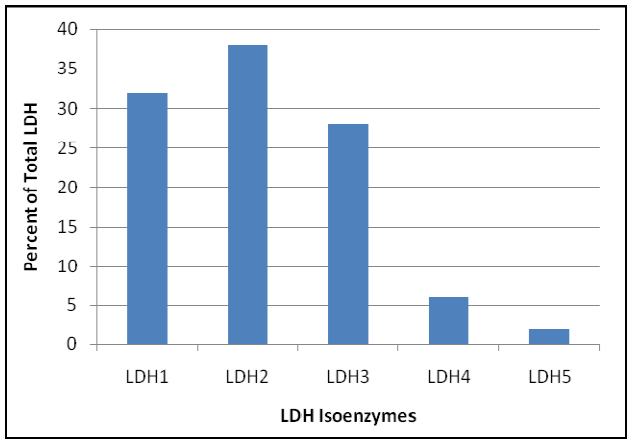

Figure 2. LDH isoenzymes pattern in meningitis patients.

The CSF-LDH level in tuberculous meningitis is ranged from 95-250 IU/L with mean of 154.24 ± 36.66, which is statistically highly significant than control (P<0.0001). The protein level in tuberculous meningitis is

ranged from 110-240 mg/100 ml with mean of 170.6 ± 36.64, which is statistically highly significant than control (P<0.0001). The sugar level in tuberculous meningitis is ranged from 20-50 mg/100 ml with mean of 39.48 ± 9.22, which is statistically highly significant than control (P<0.0001) (Table 3).

In Table 4 shows that CSF-LDH level in viral meningitis is ranged from 22-73 IU/L with mean of 49.58 ±15.58, which is statistically highly significant than control (P<0.0001). The protein level in viral meningitis is ranged from 45-80 mg/100 ml with mean of 63.75 ± 10.25, which is statistically highly significant than control (P<0.0001). The sugar level in viral meningitis is ranged from 52-98 mg/100 ml with mean of 66.25 ± 12.07, which is statistically highly significant than control (P<0.0001).

Table 2. Comparison among CSF - LDH, protein and sugar level in cases of pyogenic meningitis with control.

Group	LDH IU/L				Protein mg/100 ml				Sugar mg/100 ml			
	Mean	SD	't' Value	P value	Mean	SD	't' Value	P value	Mean	SD	't' Value	P value
Control	31.0	9.47	20.67	0.0001	29.90	5.26	36.02	0.0001	65.65	5.48	75.46	0.0001
Pyogenic meningitis	247.65	37.58	25.05	0.0001	226.95	138.47	7.86	0.0001	23.87	8.98	12.76	0.0001

Table 3. Study showing relationship among CSF - LDH level and other parameters in tuberculous meningitis with control.

Group	LDH IU/L				Protein mg/100 ml						Sugar mg/100 ml	
	Mean	SD	't' Value	P value	Mean	SD	't' Value	P value	Mean	SD	't' Value	P value
Control	31.0	9.47	20.67	0.0001	29.90	5.26	36.02	0.0001	65.65	5.48	75.46	0.0001
Tuberculous meningitis	154.24	36.66	14.99	0.0001	170.6	36.64	23.27	0.0001	39.48	9.22	21.46	0.0001

Table 4. Table showing the relationship of CSF - LDH and other parameters in cases of viral meningitis with controls.

Group	LDH IU/L				Protein mg/100 ml				Sugar mg/100 ml			
	Mean	SD	't' Value	P value	Mean	SD	't' Value	P value	Mean	SD	't' Value	P value
Control	31.0	9.47	20.67	0.0001	29.90	5.26	36.02	0.0001	65.65	5.48	75.46	0.0001
Viral meningitis	49.58	15.58	11.04	0.0001	63.75	10.25	21.54	0.0001	66.25	12.07	19.04	0.0001

The LDH level did rise quite significantly in pyogenic meningitis (Mean 247.65 IU/L Range 181-333 IU/L and p<0.0001). In control group the range of CSF-LDH was 10-44 I.U./L with a mean of 31.0± 9.47 I.U./L (Table 2). It was almost concluded that the estimation of CSF-LDH is of diagnostic as well as prognostic value particularly if interpreted together with clinical examination and routine cytochemical examinations. In cases of tuberculous meningitis also the CSF-LDH level was significantly high but less than that of pyogenic meningitis (Range 95-250 IU/L, Mean 154.24 IU/L, p<0.0001). In tuberculous meningitis also CSF-LDH estimation is of diagnostic and prognostic importance. In viral meningitis the CSF-LDH levels was slightly higher than that of normal and significantly lower than that of tuberculous meningitis and pyogenic meningitis (Range 22-73 IU/L, Mean 49.58 IU/L, S.D. 15.58 IU/L and S.E.M. 4.49 IU/L shown in Table 1. In viral meningitis CSF-LDH estimation may differentiate it from that of tuberculous and pyogenic meningitis and so of diagnostic importance.

DISCUSSION

The meningitis is one of the important causes of considerable morbidity and mortality in children's. In order to differentiate aseptic meningitis to the bacterial meningitis, numbers of studies have shown the effectiveness of rapid and definite tests using CSF variables and markers of peripheral blood for various common and uncommon laboratory measurements [12-14]. This observation is quite in accordance with the observations made earlier by Sharma et al. [15]; Nussinovitch et al. [16] who also observed raised LDH

level in the CSF of patients of pyogenic meningitis. Several conditions are known to modify the normal CSF LDH isoenzyme distribution, including bacterial meningitis (increase in LDH-4 and LDH-5), viral meningitis (increase in LDH-1 and LDH-2), hydrocephalus (LDH-2 and LDH-3) intracranial tumours (LDH-5), cerebral haemorrhage (LDH-3, LDH-4 and LDH-5), leukaemic and lymphomatous infiltration (LDH-3 and LDH-4), and tuberculous meningitis (LDH-3) [17]. Some researchers have suggested a disturbance in the blood-brain barrier which enables plasma LDH to reach the CSF, or production of LDH by neoplastic tissue or by white blood cells and exogenous bacterial sources [17-19]. In viral meningitis CSF-LDH estimation may differentiate it from that of tuberculous and pyogenic meningitis and so of diagnostic importance.

So CSF-LDH estimation is of importance as a diagnostic and prognostic tool as far as the dreaded disease of different types of meningitis are concerned.

CONCLUSION

It is conclude that biochemical profile of CSF variables have shown the effectiveness of rapid and definite tests for meningitis and treatment. However, this increase in protein level is due to the increased membrane permeability which may lead to increase CSF enzymes proportionately, which help to rule out in the differential diagnosis of meningitis.

AUTHORS CONTRIBUTION

All authors have equally contributed. The final manuscript has been read and approved by all authors.

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SHORT COMMUNICATION

Comparison of protein isolation methods from clear cell Renal Cell Carcinoma tissue

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ABSTRACT

This study presents the comparison of three protein extraction methods in the investigations of clear cell Renal Cell Carcinoma tissue. For protein isolation, we applied: phenol extraction according to Hurkman and Tanaka (1986) protocol (method 1), whole tissue lysis in urea-containing buffer (method 2) and commercially available protein isolation kit (2-D Clean-up Kit) (method 3). Statistical analysis indicated that the additional preparation steps including extraction and purification of proteins by 2-D Clean-up Kit significantly increased the quality of obtained data. We believe that gathered information could be a valuable lead for researchers involved in proteomic studies of renal tissue.

Keywords: Biomarkers; Clear cell Renal Cell Carcinoma; Protein isolation; Proteomics; Renal tissue.

INTRODUCTION

Renal cell carcinoma (RCC) is still a major problem in modern oncology. Even though, it accounts for only 3% of cancer diagnosis, there are still more than 100 000 deaths per year worldwide [1]. Approximately 80% of RCCs are classified as clear cell carcinomas. Unfortunately, currently applied treatments are not efficient enough, especially in cases of advanced cancers. Early diagnosis of RCC significantly increases the 5-year survival rate (~85%) in comparison with those detected at metastatic stage (~9%). Thus, there is a large need for discovery of selective, sensitive and easily-accessible RCC biomarker [2]. Two-dimensional electrophoresis coupled with mass spectrometry is often applied strategy in biomarker studies in order to reveal potential disease-associated diagnostic proteins. Proteomics has shown great potential in renal research, however, it also has some serious limitations related to difficulties in identification of membrane and low abundance proteins, which possibly could serve as diagnostic biomarkers. In addition, currently used methods for protein isolation cause high protein loss, and therefore, proteome maps does not represent the whole set of renal proteins. Hence, optimization of the protocols for protein isolation, solubilization, purification and separation is crucial in increasing of protein yield, resolution of polyacrylamide gels and consequently the chances of finding a marker [3]. Our investigation has focused on the comparison of different protein isolation protocols in studies of RCC biomarkers. We applied 3 protocols: phenol extraction according to the Hurkman and Tanaka (1986) (method 1), whole tissue lysis in urea-containing buffer (method 2) and commercially available protein isolation kit (2-D

Clean-up Kit, GE Healthcare, Little Chalfont, UK) (method 3).

1 cm^3 block of normal renal cortex and adjacent cancerous tissue (without surrounding fibroadipose tissue) was removed *ex vivo* after radical nephrectomy and was examined by uropathologist according to Fuhrman grading system and American Joint Committee on Cancer clinical staging system. Final pathological report confirmed malignant character of tumor: clear cell RCC, Fuhrman III, pT1b (5.5 cm) with invasion of tumor beyond renal capsule into perinephric fat. The samples were immediately frozen in liquid nitrogen and stored at -80 °C until use.

Frozen renal cortex was grounded in a mortar with liquid nitrogen and acquired powder was divided into 3 equal parts. First portion was extracted in accordance with the Hurkman and Tanaka protocol (method 1) [4]. The powdered tissue was solubilized in 500 µl of the extraction buffer (0.7 M sucrose, 0.5 M Tris, 30 mM HCl, 50 mM EDTA, 2% DTT, and 0.1 M KCl). The same amount of Tris/EDTA-saturated phenol was then added and the sample was incubated for 5 min at 4 °C. After that, the sample was vortexed by 10 min and centrifuged at 8 700 G for 30 min. The upper phenol phase was collected and re-extracted in 500 µl of the extraction buffer. After second centrifugation, 5 volumes of cold 0.1 M ammonium acetate in methanol was added to the phenol phase to precipitate the proteins. Sample was then incubated at -20 °C overnight and centrifuged at 20 500 G in 4 °C for 30 min. The precipitate was washed twice with the cold ammonium acetate in methanol and once in cold acetone, and dried. Obtained pellet was finally dissolved in 500 µl of the sample solution (7 M urea, 2 M thiourea, 2% NP-40, 2% IPG buffer pH range 3-10, 40 mM DTT).

Remained fractions were homogenized in 500 µl of the sample solution each, vortexed and sonicated for 10 min. The samples were centrifuged at 8 700 G in 4 °C for 30 min (method 2). Acquired supernatants were collected and placed into new tubes. Half of the sample was prepared using commercially available 2-D Clean-up Kit (GE Healthcare). Protein purification step was done according to procedure B from the protocol brought by manufacturer (method 3).

The concentration of proteins in samples was measured using the 2-D Quant Kit (GE Healthcare). 550 µg of the proteins was loaded onto 24 cm Immobiline DryStrip gels (GE Healthcare) with linear pH range 3-10 to perform isoelectrofocusing. The process was conducted in Ettan IPGphor II (GE Healthcare) using 2 µA per strip at 20 °C. Whole program was divided into 12 h of active rehydration (20 V) followed by 10 h of focusing (1^{st} h - 500 V; 2^{nd}-3^{rd} h - 1 000 V; 4^{th}-6^{th} h - 8 000 V; 7^{th}-10^{th} h - 10 000 V). After equilibration, second dimension (SDS-PAGE) was performed on 13% polyacrylamide gels (1.5 × 255 × 196 mm) with Roti®-Mark PRESTAINED protein molecular weight marker (Roth, Karlsruhe, Germany). The electrophoresis was carried out on EttanDalt Six (GE Healthcare) for 5.5 h (0.5 h - 4 W per gel, 5 h - 17 W per gel). The protein spots were stained with colloidal Coomassie Brilliant Blue G-250 according to Neuhoff protocol [5].

The gels were scanned by ImageScanner III (GE Healthcare) and processed by LabScan 6.0 (GE Healthcare). Image Master 2-D Platinum software (GE Healthcare) was used to manually pick and count the number of spots on gels.

For Student's t-test, the means and standard deviations of the spot number from three independent replicates were compared between 3 gels of the ccRCC and 3 gels from the control for each isolation method by applying the standard algorithm [6]. The level of significance was set to $p < 0.05$ and data were compared by using Welch's t-test (because the variances of two populations are not equal). The differences between the methods were analyzed by one-way analysis of variance using appropriate tests [7] in STATISTICA (StatSoft, Inc., Tulsa, Oklahoma).

The comparison of three protein isolation methods was performed in order to assess their effectiveness in the studies of ccRCC proteome. Isolation and purification of proteins by 2-D Clean-up Kit led to the best clarity and resolution of the electrophoretic gels after 2DE. In contrast, the protein separation after lysis in urea-containing buffer provided less transparent gels (Fig. 1).

The statistical analysis revealed that in the investigation of healthy tissue, application of method 3 gave the highest number of spots on gels (~899), whereas method 2 provided the least spots (~694). One-way analysis of variances confirmed that differences between all three methods in the case of non-cancerous tissue were statistically significant (Fig. 2). Methods 1 and 3 showed high variation level of spot number between control tissue against sample tissue. Student's t-test confirmed the significance of these differences ($p < 0.05$). In turn, the gels from method 2 contained more protein spots on sample than on control (Fig. 3). However, the difference here was non-significant. Regarding to ccRCC samples, methods 2 and 3 gave similar yield, both significantly different from method 1 (Fig. 2).

The aim of this research was to compare the methods of protein isolation in proteomic studies of renal tissue based on 2DE separation. Efficient protein isolation and purification steps are crucial in future investigations, especially in the case of biomarker discovery research. Thus, selection of the most suitable method could significantly increase probability of success.

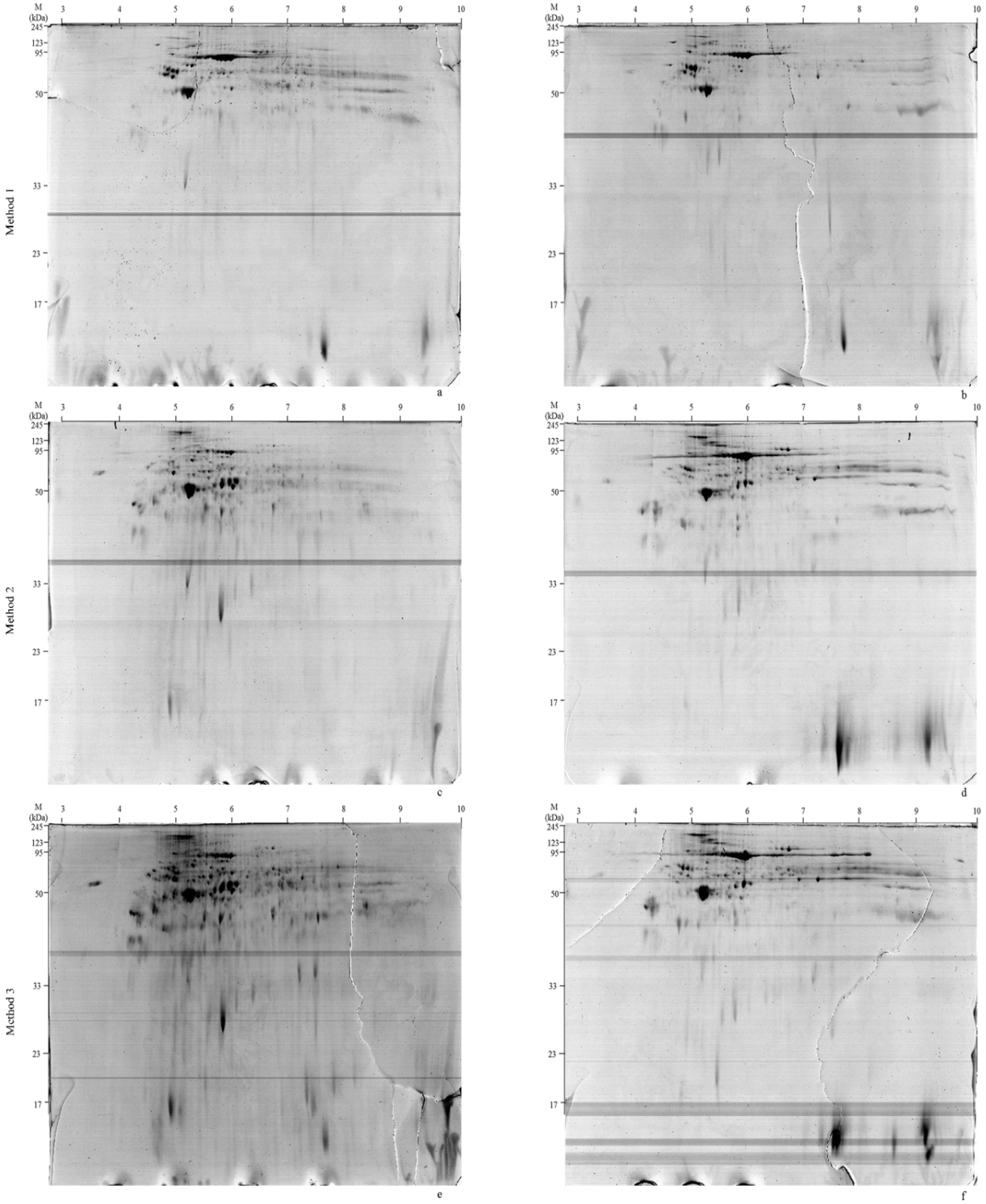

Figure 1. Representative 2-D protein maps obtained from healthy (left) and malignant renal tissue fragments (right) of patient with diagnosed ccRCC. The figure shows the differences in clarity and resolution of gels after 3 different protein isolation methods.

The comparison of 3 methods was performed to evaluate their usefulness in proteomic research of ccRCC biomarker exploration. The conclusions from this study are based on statistical analysis of spots as well as on visual assessment of gels. Previously described biomarker research were often conducted using whole cell lysate without any further isolation and purification steps [8-11]. However, our results indicate that

application of methods 1 and 3 are the best for comparative studies due to the high level of variation between healthy and cancerous tissue. Method 3 provided satisfactory number of spots on gels of both tissue types, most likely due to the lower protein loss. Our research demonstrated that isolation and purification of proteins using commercially available 2-D Clean-up Kit could improve the value of the obtained data and therefore increase chances of potential biomarker discovery.

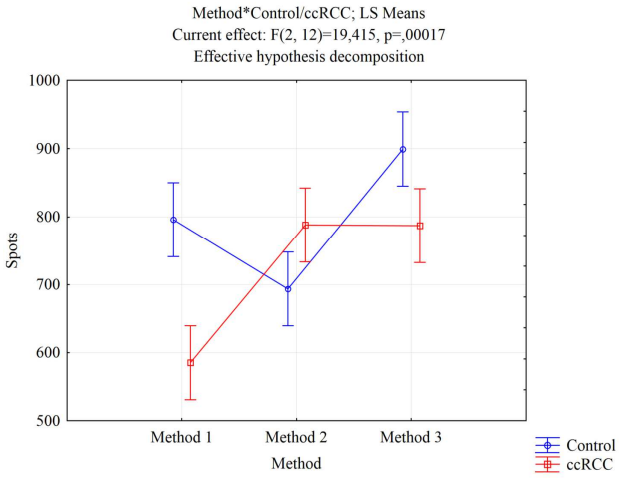

Figure 2. Analysis of variance (ANOVA) shows the average difference in the amount of spots between methods.

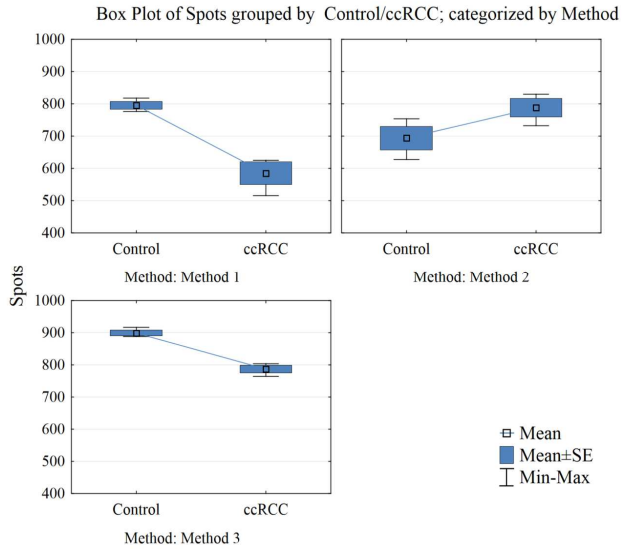

Figure 3. The comparison of differences in spot number between healthy (control) and malignant tissue (ccRCC) within each method.

The results also indicate that despite the high content of fat in ccRCC tumors [12], method 1, which is designated for the hydrophobic proteins, has not increased the amount of isolated protein. We expected that this method of extraction may allow for the isolation of proteins with less hydrophilic character, however this did not happen and the number of spots on the gels obtained from cancer tissue by this method was the lowest. This may indicate that the observed fat deposition acts rather as the cell insulator than as the integral organ tissue with its own protein profile.

In summary, our study provides data on protein isolation efficiency in the investigations of renal tissue based on 2DE. We believe that gathered information could be a valuable lead for researchers involved in such studies.

AUTHORS CONTRIBUTION

Conceptualization: AB, AŁ; Formal analysis: KKH, MJ, AB; Investigation: KKH, AB; Project administration: GG, ZD; Resources: KO, TO, AO, AB, AŁ, KKH; Supervision: AB, AŁ; Original draft preparation/review and editing: KKH, AB, AŁ, KO. The final manuscript has been read and approved by all authors.

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RESEARCH ARTICLE

Women are more susceptible than men to micronutrient depletion during petrol exposure

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ABSTRACT

The influence of gender on the modulating effects of many xenobiotics has been suggested. The aim of the study is to determine which of the genders, is more at risk of altered micronutrient status subsequent to petroleum-product exposure. 60 petrol-station attendants (PSA) as well as 60 control participants were recruited for the study. The test or control group for each gender consisted of 30 participants. Serum obtained from five millimeters of blood was used to determine the levels of micronutrients. Vitamins and trace elements were determined using High Performance Liquid Chromatography and Atomic Absorption Spectrometry respectively. Significant differences between groups were ascertained using Student's t test. Correlation study between length of service (at petrol station) and micronutrient levels was determined using Pearson's correlation co-efficient. $P<0.05$ was considered significant. Although correlation was observed between length of service and several micronutrients for both male and female PSA and petrol caused significant reduction in micronutrient levels in both groups of PSA; Mo, Fe and Se, pyridoxine, niacin, vitamins E and vitamin C were more significantly lower in female PSA than male ones. These results suggest that women are more susceptible than men to micronutrient depletion during petrol exposure.

Keywords: Male; Female; Vitamins; Minerals; Petrol station attendants.

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INTRODUCTION

A number of chemicals have been identified that alter levels of essential vitamins and minerals in the human body [1]. While some of them are household products that are required for every day existence; some others are of therapeutic importance [2, 3]. Gasoline is a modern day necessity. Petroleum products are employed as fuel for industrial use as well as to power automobiles or other machines [4]. Several millions of litres of gasoline are sold every day in Nigeria. The properties of its components make contact with humans unavoidable. Gasoline is a refined product of crude oil that contains more than 150 hydrocarbons with a range of boiling point from 40°C to 180°C [5]. Hydrocarbon content of gasoline include alkanes (paraffins), isoparaffins, alkenes (olefins) and naphthenics, its aromatic compounds are benzene, toluene, ethylbenzene and xylene [5]. Many of these components are known to be volatile [6].

Slight differences in physiology have been ascribed to be the basis of great variations in response to xenobiotic exposure even within the same species. The male hormone testosterone has been identified to enhance the toxicity of high doses of acetaminophen [7]. In

addition, a more pronounced hepatotoxic and carcinogenic effect of aflatoxin B1 in males than females is well documented. Such that in parts of the world where aflatoxin B1 contamination of crops is common and it has been identified as a casual agent that synergistically interacts with hepatitis B virus infection in the pathogenesis (etiologic factor) of hepatocellular carcinoma, the incidence of hepatocellular carcinoma is in the ratio of 4: 1 in favor of women [8]. A higher level of oxidative stress has also been recognized in female subjects exposed to polycyclic aromatic hydrocarbons when compared with males [9].

Many of the components of antioxidant defense system (including antioxidant micronutrients) are sometimes altered in human subjects as a result of oxidative-stress induced xenobiotic administration. The objective of this study is to determine the deleterious effect of petroleum product exposure on serum micronutrient levels of male and female PSA as well as investigate which of the two groups is more susceptible to such exposure.

MATERIALS AND METHODS

Subjects

Being a pilot study, the sample size of the studied population was 60 persons (30 participants for each gender). Adults above the age of 19 years, who have worked consistently in petrol station for at least 5 years, with between 8-10 hours of daily work shift were recruited for the study. Not more than 2 petrol station attendants (PSA) were recruited from any petrol station; none of the stations captured in the study had less than 10 attendants on their payroll. Random sampling technique was employed not only to recruit petrol station attendants that met the inclusion criteria, but also used in selection of the petrol stations captured in the study.

Individuals were assisted in filling questionnaire and the information provided was used in selecting suitable participants that met the inclusion criteria. The questionnaire was constructed to obtain information on their socio-economic/demographic status (age, gender, income, educational level); medical state (disease, medication) lifestyle (smoking pattern, alcohol consumption, etc); as well as occupational information (duration of exposure to petroleum products/length of service at petrol station, number of shift per week, number of hours per shift). Equally information was obtained about use of protective gears (face mask, gloves, overall). Confidentiality was maintained with regard to the information provided by the respondents.

A total of 60 participants (30 for each gender) above the age of 19 years were also selected from the general population. They had not been involved in any occupations associated with exposure to gasoline/diesel (e.g. automobile mechanic workshops or automobile fuelling stations). Because of the volatile nature of gasoline and since many of the filling stations are located in residential quarters, during subject recruitment process; every effort was made to ensure that control subjects were not living in very close proximity to petrol/filling stations. In Nigeria, it is a common knowledge that during petroleum product scarcity, these products are bought into containers to be emptied into fuel tanks of automobiles/motorcycles, therefore commercial car drivers and motorcycle riders were also excluded as control subjects. Other exclusion criteria for test and control groups include micronutrient supplementation as well as any disease capable of altering serum micronutrient levels. Moreover, lifestyle was also considered as exclusion criterion; all participants with lifestyles capable of altering micronutrient levels were not recruited for the study. Since socio-economic markers have been identified to have impact on levels of micronutrients, all participants recruited for the study were of the same socio-economic status; income, education and occupation were markers adopted for this purpose.

Informed consent was obtained from each subject prior to the commencement of the study. Five millimeters of venous blood was taken from antecubital vein of each participant and dispensed into anti-coagulant and micronutrient free bottles. They were delivered to the laboratory not more than 2 hours after collection. The samples were then centrifuged and sera stored at -20°C until the period of analysis. All samples were obtained during work shift before noon (12:00). All procedures were carried out in accordance with revised Helsinki Declaration (1983).

Micronutrient analysis

Serum levels of vitamins A, B_6, C, D and E, thiamine, riboflavin, niacin, and folic acid were determined by employing High Performance Liquid Chromatographic technique (HPLC). Quantification of serum concentrations of Zn, Cu, Se, Mn, Co, Fe, Mo, and Cr took place using the Atomic Absorption Spectrometric method. Waters® Corporation Milford, Massachusetts USA supplied HPLC equipment whereas Buck Scientific 205 Atomic Absorption spectrometery was supplied by Buck Scientific in East Norwalk, Connecticut (USA). Each of the reagents utilized in the estimation of the micronutrients was of high-purity analytical grade (Merck, Darmstadt; BDH Chemicals Ltd). The water employed for the preparation of reagents and working standard was deionized, doubly distilled and re-

deionized prior to the commencement of the experiment (Millipore Co., Bedford, MA), and had specific resistance of >3 MΩ. Working standard was made from spectrosol stock standard (1g/l) that was supplied by Buck Scientific. In addition all disposable apparatus was vigorously washed before use by immersing in concentrated nitric acid and thoroughly rinsed with the same deionized, doubly distilled and redeionized water.

Statistical analysis

All analyses were done using SPSS version 15. Significant differences between male and female PSA as well as between PSA of each gender and their respective control were determined using Student's t test. Pearson's correlation co-efficient was used for correlation analysis. A p value of ≤ 0.05 was considered significant.

RESULTS

Results of the study are presented in Tables 1-4. Shown in Table 1 are the serum levels of vitamins and minerals of male petrol station attendants (PSA) and male control subjects. Of all the micronutrients estimated only riboflavin, pyridoxine, niacin, vitamin D, Se, Cr, and Mo were not significantly different (p>0.05) in male PSA compared with control, all others (vitamins A, C, E, thiamine, folic, Zn, Cu, Mn, Co, Fe) were significantly lower (p<0.05). The results of serum micronutrients of female PSA and female control group are presented in Table 2, vitamins A, C, E, thiamine, niacin, folic, Zn, Cu, Mn, Cr, Co, and Fe were significantly lower (p<0.05) in female PSA compared with female control whereas riboflavin, pyridoxine, vitamin D, Se, and Mo were not significantly different (p>0.05). When the results of serum micronutrients of both female and male PSA were compared in Table 3; riboflavin, thiamine, vitamins A and D, as well as Zn, Mn, and Cr were not significantly different (p>0.05) in both groups whereas folic acid, niacin, vitamin C and E, Se, Fe, Mo and Co were significantly lower (p<0.05) in female PSA compared with male while pyridoxine and Cu were significantly higher (p<0.05).

The results of the personal/basic characteristics of both males and females PSA are presented in Table 4. Age, duration of employment time/length of service at petrol station(s), number of shift per week, and number of hours per shift were not significantly different for both groups. None of the PSA had used facemasks in the preceding 24 months; none of the male PSA but 3 of female PSA had utilized the protective gear- hand gloves in the past 12 months.

Table 1. Serum levels of micronutrients of male petrol station attendants and control subjects.

Micronutrients	Male Control (n =30)	Male PSA (n =30)	p-value
Riboflavin (nmol/l)	614.67±31.72	620.11± 20.83	0.702
Folic (nmol/l)	18.43±3.87	15.04±6.60	0.026[a]
Pyridoxine (nmol/l)	72.45±7.71	70.68±4.95	0.401
Niacin (nmol/l)	19.31±17.29	18.63±15.38	0.640
Thiamine (nmol/l)	144.70±22.59	130.90±30.22	0.018[a]
Vitamin D (nmol/l)	98.21±5.48	97.52±11.70	0.092
Vitamin E (µmol/l)	25.21±7.00	20.27±6.64	0.014[a]
Vitamin C (mmol/l)	59.09±6.26	51.62±9.04	0.022[a]
Vitamin A (µmol/l)	2. 01±0.16	1.70±0.10	0.009[a]
Se (µmol/l)	1.28±0.23	1.16±0.24	0.078
Cu (µmol/l)	17.06±4.37	14.55±3.85	0.019[a]
Fe (µg/dl)	144.89±16.31	131.30±31.07	0.032[a]
Mo (nmol/l)	21.00±1.87	22.26±4.48	0.384
Zn (µmol/l)	16.12±4.03	13.33±3.69	0.041[a]
Mn (nmol/l)	14.50±3.13	11.64±2.06	0.017[a]
Cr (nmol/l)	1.92±0.03	1.99±0.14	0.059
Co (nmol/l)	4.94±0.79	3.14±0.92	0.025[a]

Results are expressed as mean±standard deviation. [a] Significant difference when male petrol station attendants and control were compared. p value ≤ 0.05 was considered significant.

Table 2. Serum levels of micronutrients of female petrol station attendants and control subjects.

Micronutrients	Female Control (n =30)	Female PSA (n =30)	p-value
Riboflavin (nmol/l)	639.06±40.91	633.27±26.70	0.912
Folic (nmol/l)	17.75±3.33	12.33±3.90	0.019[b]
Pyridoxine (nmol/l)	83.30±7.98	80.39±5.43	0.162
Niacin (nmol/l)	18.74±22.82	15.85±8.17	0.038[b]
Thiamine (nmol/l)	132.99±28.07	122.41±15.40	0.040[b]
Vitamin D (nmol/l)	92.17±9.04	94.08±5.05	0.066
Vitamin E (µmol/l)	26.74±5.40	16.30±3.92	0.024[b]
Vitamin C (mmol/l)	63.67±9.39	44.01±4.48	0.011[b]
Vitamin A (µmol/l)	1.96.08±0.12	1.62±0.22	0.119[b]
Se (µmol/l)	1.30±0.45	1.00±0.09	0.051
Cu (µmol/l)	22.97±8.03	18.36±5.45	0.037[b]
Fe (µg/dl)	122.58±20.22	109.80±27.74	0.029[b]
Mo (nmol/l)	19.93±3.39	18.32±5.00	0.553
Zn (µmol/l)	15.29±6.61	12.29±4.43	0.014[b]
Mn (nmol/l)	15.91±4.06	12.77±2.93	0.013[b]
Cr (nmol/l)	1.98±0.08	1.84±0.11	0.020 [b]
Co (nmol/l)	5.57±0.52	4.41±0.50	0.012[b]

Results are expressed as mean±standard deviation. [b] Significant difference when female petrol station attendants and control were compared. p value ≤ 0.05 was considered significant.

Table 3. Serum levels of micronutrients of male and female petrol station attendants.

Micronutrients	Male PSA (n =30)	Female PSA (n =30)	p-value
Riboflavin (nmol/l)	620.11± 20.83	633.27±26.70	0.406
Folic (nmol/l)	15.04±6.60	12.33±3.90	0.049 [c]
Pyridoxine (nmol/l)	70.68±4.95	80.39±5.43	0.037[c]
Niacin (nmol/l)	18.63±15.38	15.85±8.17	0.042[c]
Thiamine (nmol/l)	130.90±30.22	122.41±15.40	0.311
Vitamin D (nmol/l)	97.52±11.70	94.08±5.05	0.172
Vitamin E (µmol/l)	20.27±6.64	16.30±3.92	0.040[c]
Vitamin C (mmol/l)	51.62±9.04	44.01±4.48	0.024[c]
Vitamin A (µmol/l)	1.70±0.10	1.62±0.22	0.533
Se (µmol/l)	1.16±0.24	1.00±0.09	0.037[c]
Cu (µmol/l)	14.55±3.85	18.36±5.45	0.042[c]
Fe (µg/dl)	131.30±31.07	109.80±27.74	0.033[c]
Mo (nmol/l)	22.26±4.48	18.32±5.00	0.014[c]
Zn (µmol/l)	13.33±3.69	12.29±4.43	0.310
Mn (nmol/l)	11.64±2.06	12.77±2.93	0.199
Cr (nmol/l)	1.99±0.14	1.84±0.11	0.701
Co (nmol/l)	3.14±0.92	4.41±0.50	0.113[c]

Results are expressed as mean±standard deviation. [c] Significant difference when male and female petrol station attendants were compared. p value ≤ 0.05 was considered significant.

Table 4. Personal/basic characteristics of petrol station attendants (female and male) and control.

	Male PSA (n =30)	Female PSA (n =30)	P value
Age (years)	26.90±4.2	28.20±5.3	0.471
Duration of employment at petrol station (years)	5.75±1.0	6.25±2.2	0.339
Number of shifts per week	6.50±0.9	6.10±1.2	0.720
Number of hours per shift	9.50±2.1	8.60±3.8	0.096

Results are expressed as mean±standard deviation. p value ≤ 0.05 was considered significant.

These 3 female did so because of dermal sensitivity to petrol; they revealed that contact with petrol resulted in skin irritation, even then hand glove was infrequently employed. All PSA reported infrequent use of overall although its frequency of use was higher in female than male PSA. In addition, in stations where overall were not provided but petrol station uniform made available, more females employed the use of work uniform than males. This was not considered as overall. In female PSA correlation was noted between length of service at petrol station and zinc ($r = -0.419$, $p = 0.024$), Cr ($r = 0.382$, $p = 0.045$), Co ($r = -0.477$, $p = 0.010$), and folic acid ($r = -0.581$, $p = 0.038$) but only zinc ($r = -0.371$, $p = 0.049$), Cu ($r = 0.609$, $p = 0.006$), and folic acid ($r = -369$, $p = 0.045$) were correlated with length of service at petrol station in male PSA.

DISCUSSION

Self-servicing at fuel filling station is common in industrialized parts of the world, but this is not so for many developing nations where the pumps are manned by attendants. Because of the nature of petroleum products, especially its volatile property, exposure of their toxic constituents to people within petrol station vicinity cannot be ruled out. Some job types, and life-style as well as exposure to toxic and therapeutic agents are known to present with gender biased toxic response. The study of Hu et al. [10] gives an insight into a case of sex/gender bias in the disposition of an experimental animal to toxic agent exposure. Male mice were found to be more susceptible to paracetamol toxicity than female ones. Hu et al. [10] hypothesized that that was possible because of the modulating role of testosterone in paracetamol-induced toxicity. A hypothesis that was confirmed when testosterone pretreated paracetamol dosed female rats exhibited the same degree of toxicity as male rats given the same dose of paracetamol. Yet it is not only sex hormones that have been linked with differences in gender response to a toxic agent, the cytochrome P450 enzymes also have been implicated in such result outcomes. The kind of data obtained from this study; in which female petrol station attendants featured a more significant decrease in the serum levels of many of the micronutrients than their male counterparts, is an indication that females may be more susceptible to toxic effects of gasoline exposure than males.

The constituents of crude oil and ultimately its products that the attendants are exposed to are benzene, toluene, etc. [5]. Benzene especially has been identified to be metabolized through the cytochrome P450 pathways to yield highly toxic and reactive metabolites such as benzene oxide and phenol. This may be a cause of the low levels of many of these micronutrients observed in the two categories of PSA. Many of the micronutrients possess antioxidant properties; ascorbic acid has been recognized for its notable function in regeneration of glutathione i.e. the conversion of oxidized glutathione (GSSG) to its reduced form (GSH). There is every possibility that decreased ascorbic acid level observed in PSA occurred from increased demand of the antioxidant property of GSH. Vitamin E on the other hand plays an important role in preventing lipid peroxidation. A number of essential elements (especially Zn, Cu, Mn) estimated in serum of PSA that were found to be significantly decreased have been linked with the antioxidant enzyme superoxide dismutase. An enzyme that plays vital roles in conditions of increased oxidative stress.

Occupation as a cause of infertility or sub-fertility has been observed in many different classes of artisans but Bull et al. [11] reported that paternal exposure to hydrocarbons in the occupations studied did not seem to have had a major influence on time to conception or the incidence of spontaneous abortion among the wives of the men exposed to oil products. While zinc is essential in the process of spermatogenesis, low levels of maternal folic acid which to a great extent has been linked with neural tube defects in neonates was significantly lower in female petrol station attendants compared with their male counterparts. A good percentage of female PSA are known to be within reproductive stage. The fact that serum levels of antioxidant micronutrients (Cu Zn, vitamins C, E) of male PSA were also significantly decreased, means that male PSA may also have increased risk to several oxidative stress related diseases but the since depletion was intensified in female PSA than their male counterparts, females therefore may have elevated

risk compared with males. Supporting the possibility that petrol exposure may adversely affect the health of individuals is that not only were zinc and folic acid (essential micronutrients at all stages of human development) significant lower in both groups of PSA when compared to their respective controls, both were also negatively correlated with length of service in PSA.

While this study has provided biochemical evidence which indicates that the consequences of petrol exposure is more grave in females than males; the cause of the significant differences in micronutrient levels of both male and female PSA may not be unassociated with dissimilarity in the physiologic make-up of both sexes, examples being sex hormone levels or differences in expression of cytochrome 450 isozymes. A wide range of microsomal enzymes (CYP2E1, CYP2F2, CYP2A6, etc) have been recognized to play a role in metabolism of constituents of petroleum products [12, 13] yielding highly reactive species capable of modulating levels of biomolecules with antioxidant potential. Moreover, it should not be surprising that the levels of Cu, vitamins C and E were lower in female PSA compared with male ones, since the expression levels of some cytochrome 450 isozymes are generally higher in females than male [14].

Even though it is speculative to suggest that either sex hormone or differences in cytochrome 450 may be responsible for differences in micronutrient presentations of both male and female PSA (as neither was included in the study), it should be worthwhile to investigate what inherent difference in male and female PSA is responsible for variations in serum micronutrient levels observed when both groups were compared. Street trading and hawking is very common in Nigeria, the use of pre-pubertal male and female hawkers who are constantly found in the filling station may help to achieve this aim. In addition such subjects can serve for longitudinal study to assess long time effect of early exposure to petroleum products.

While this study was carried out on those with closest proximity to petroleum products, filling stations are indiscriminately sited in many of the major cities in Nigeria, it can be deduced from the data obtained from the study, that female subjects who live for extended period of time in the same neighborhood as these stations may present with depletion in serum micronutrient levels. Various past studies suggest this possibility; contact with benzene (a significant component of petrol) has been reported to cause damage to DNA in traffic policemen [15], petrochemical workers [16], shoe makers [17], and printing company workers [18]. The use of protective gears has been advocated in various occupations to diminish hazard that may result in injury or illness and there is evidence to prove that if strictly adhered to, it may result in considerable protection. Since there was general non-compliance with regard to use of protective gears by both male and female PSA, it may be postulated that such attitude contributed to the gravity of micronutrient depletion observed in both groups.

The implication of results of this kind is diverse. Essential trace elements are known for their roles as co-factors of enzymatic reactions in hundreds of chemical processes. One of the notable roles of zinc (observed to be significantly decreased in both male and female PSA) is its function in DNA repair system; the fact that this important function may be compromised by exposure to petroleum product may be postulated from results obtained through various past studies. Different markers of DNA damage have been shown to be gravely affected by petrol exposure Using different biomarkers, such as sister chromatid exchange (SCE) or DNA strand break, Kanupriya et al. [19] demonstrated an elevation in degree of cytogenetic damage in peripheral blood lymphocytes of workers exposed to gasoline. There was also significant difference in chromosomal abnormalities between petrol-exposed workers and control especially with respect to mitotic index [20]. In addition, buccal smears collected from Nigerian petrol station pump attendants chronically exposed to petrol fumes and control, subjected to micronucleus assay showed a significant difference in micronuclei detection between the exposed and control groups, suggestive of DNA damage in petrol station pump attendants [21]. Summing up the results of many studies, Mutgud et al. [22] noted that there were increase frequency of DNA and chromosomal damage in human population exposed to petroleum products. Also according to them, mutagenic and carcniogenic effects of diesel and petroleum vapors have been reported in not only human subjects but also different biological models.

CONCLUSION

Results of this study suggest that PSA are at risk of micronutrient alteration. In addition, it seems evident that females are more at risk than males. If left unattended, exposure to petrol may increase an individual's risk to micronutrient-related diseases.

AUTHORS' CONTRIBUTION

Authors contributed equally with respect to experi-mentation, data analysis and interpretation, and preparation of manuscript. The final manuscript has been read and approved by both authors.

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RESEARCH ARTICLE

Extract of *Moringa oleifera* attenuates hematological parameters following salt loading

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ABSTRACT

Moringa oleifera Lam. is an important plant with huge medicinal potentials. This study is aim at investigating the effect of leaf extract of *Moringa oleifera* on some haematological parameters in salt loaded albino Wistar rats. A total of twenty four (24) male albino Wistar rats weighing between 200 to 250 g were used for the experiment and were divided into four groups of six rats each. They were given either normal rat feed and drinking water, high salt diet (8% NaCl diet) + 1% NaCl drinking water and/or *Moringa oleifera* extract (600 mg/kg b.w., orally, once daily). The feeding regimens lasted for six weeks. After an overnight fast, blood was collected and analyzed for hematological parameters. The salt fed untreated rats had significant ($p<0.05$) increase in red blood cell count (RBC) and packed cell volume (PCV), ($P<0.01$). These parameters were significantly ($p<0.05$) reduced to near control values following extract treatment. Salt fed untreated rats were observed to have significant ($p<0.05$) reduction in total white blood cell count (TWBC) and platelet (PLT) count ($P<0.01$), but the reverse was the case following extract treatment. In conclusion consumption of *Moringa oleifera* leaf extract ameliorates the adverse effects of salt loading on the blood cells. Therefore, the extract is recommended to pharmaceutical industries for further research and possible use in the manufacture of drugs that are necessary in management of blood pressure and other related ailments.

Keywords: *Moringa oleifera* Lam.; Hematological parameters; Rats.

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Ethical approval: Ethical approval was obtained from the Ethics Committee of the Faculty of Basic Medical Sciences, University of Calabar, Nigeria.

INTRODUCTION

The quest for human survival, wellbeing and health care led to the recognition and discovery of many plants with medicinal value before the advent of orthodox medicine. These plants are said to possess components that are of therapeutic values [1, 2]. Among these many plants, there exists one that has almost all the nutritive values required by man. This plant is usually known in most literature as *Moringa oleifera*. The tree is native to India, it also occur in different places which include Sri-Lanka, Thailand, Pakistan, Phillipine, Indonesia, Taiwan, Haiti, South America, Caribbean and Africa (Nigeria) [30]. It is commonly known as Drumstic [3] and has several other names which include horseradish tree, ben oil or benzoil tree [31], mothers best friend and Nebeday. There exist 13 species of *Moringa* belonging to Moringaceae family [4, 5].

Almost all the plant parts contain substances that are important for the synthesis of useful drugs [6] that can serve medicinal purposes, they also have important agricultural, commercial and economical values. The nutritional values of *Moringa oleifera* cannot be underestimated this is because proximate and

phytochemical analysis of *Moringa oleifera* leave extract [7] reveals that it contain important bioactive components which include carbohydrate, fat, protein and minerals eg. calcium, iron, magnesium, manganese, phosphorus, potassium, sodium, zinc and even water [8, 31, 32]. Others include vitamins (Vit. B_1, B_2, B_3, B_5, B_6, B_9, Vit. C. Vit. E and Vit. K), Carotenoids and antioxidants which include [7], flavonoids, glycosides, terpinoids, zeatin, quercetin and kaempferol [31, 32].

The antioxidant components of *Moringa oleifera*, basically Vit. C, carotene and quercetins are known to play major role in lowering blood pressure [33-35], quercetins and flavonoids can inhibit the production of nitric oxide and tumor necrosis factor by Kupffer cell when stimulated by injury [42], flavonoids also protect the cell against injury caused by x-ray and block the progression of the cell cycle and prostaglandin synthesis thereby inhibiting mutation and preventing carcinogenesis in experimental animals [43], the Vit. E antioxidant which is composed of tocopherol and alpha tocopherol is the most abundant and active component of this plant. This vitamin prevents lipid peroxidation chain reaction generated by free radicals from cellular and subcellular membrane which are rich in polyunsaturated lipid thereby preventing atherosclerosis and cancer [44, 45]. The Vitamin C components of *Moringa oleifera* can act as a scavenger of free radicals and do also regenerate Vitamin E indirectly [44], by virtue of this synergy, both vitamins C and E have attracted interest as agent that can retard atherosclerosis by reducing low density lipoproteins oxidation and thus preventing injury to the vascular endothelial cells [48]. Vitamin A is important for normal vision in dim light and for resistance against infection [13, 44, 47], the chlorogenic acid component of *Moringa oleifera* helps in moderating blood sugar level [36], the isothiocyanate component of *Moringa oleifera* also plays a major role in reducing blood sugar level in addition to its anti-inflammatory [9, 37, 38], anticancer and antimicrobial effect [39-41]. *Moringa oleifera* extract are also known to possess antitumor and hepatoprotective activities [9, 10], antispasmodic [5, 11, 12] and antiepileptic activities [14]. The antioxidant components of *Moringa oleifera* has been shown to protect against structural defect and also inhibit free radicals formation [15]. In addition, blood parameters like packed cell volume (PCV), white blood cell (WBC) counts, hemoglobin (Hb) and platelets (Plt) were shown to be enhanced following consumption of *M. oleifera* [16].

Taking cognizance of the enormous health benefit that abound resulting from consumption of *M. oleifera*, extract it is therefore very necessary to investigate the effect of this plant on hematological parameters following salt loading, thus the aim of the study.

MATERIALS AND METHODS

Experimental animals

Twenty four (24) male albino Wistar rats weighing initially between 200 to 250 g obtained from the animal house of the Department of Physiology, University of Calabar, Nigeria were employed for this study for 6 weeks. The animals were allowed free access to their feed and drinking water. The rats were weighed before commencement of the feeding experiment and thereafter were weighed daily. Ethical approval was obtained from the Ethics Committee of the Faculty of Basic Medical Sciences, University of Calabar, Nigeria. They were nursed under control of environmental conditions in accordance with international standard [29].

Moringa oleifera extract preparation

The aqueous extract was prepared according to standard method [17]. Fresh leaves of *Moringa oleifera* were obtained from Calabar municipality, Cross River State and were identified by the Herbarium in Botany Department. The leaves were washed to remove debris and were later dried in an airy-room away from direct sunlight to avoid possible damage to their phyto-constituents for two days. The leaves were further oven-dried for 30 minutes at the temperature of 40^0C. The dried leaves were grinded to powder form.

About 1400 g of the powdered *Moringa oleifera* leaf was soaked in 7000 ml of distilled water for about 24 hours. The mixture was then filtered with a white cotton (satin) material, followed with filter paper (Whatmann No.1) into beakers and placed in an oven. The filtrate was evaporated to dryness using a rotary evaporator with temperature set at 50^0C. *Moringa oleifera* extract was then collected into a sample bottle and preserved in a refrigerator.

Acute toxicity test (lethality study)

Acute toxicity test was done according to standard procedure [18]. Thirty six mice rats were used for the study. They were randomly selected and assigned to six batches containing six animals each. They were allowed a week for adaptation. Each batch received doses of extract intraperitoneally (1.64-104.48 mg/kg). Only the control group received normal saline intraperitoneally. They were all returned to their home cages and allowed free access to food and drinking water. The mortality in each group was assessed 24 hours after administration of the extract. The percentage mortalities were converted to

probits and plotted against the $\log_{10}$of the dose of the extract [18].

Salt diet and drinking water

Salt feed containing 8% NaCl was prepared by putting together 8 g NaCl in 92 g of the rat feed. Also 1% NaCl drinking water was prepared by dissolving 100 g of NaCl in small quantity of distilled water and volume made up to 10 l with distilled water.

Experimental design

Twenty four (24) male albino Wistar rats weighing between 180-240 g was randomly assigned into four (4) groups of six (6) rats each:
Group 1 (control): received normal rat feed + drinking water
Group 2: received same as group 1 + *Moringa oleifera* extract (600 mg/kg o.p. once daily)
Group 3: received 8% NaCl diet + 1% NaCl drinking water
Group 4: received same as group 3 + *Moringa oleifera* extract (600 mg/kg o.p. once daily).

The administration was done orally and the experiment lasted for a period of six weeks. Also the Helsinki declaration of 1975 as revised in 1985 was strictly adhered to.

Collection and analysis of hematological parameters

Blood samples were collected via cardiac puncture into EDTA capped bottles and the full blood analysis was done using automated hematology analyzer Sysmex model: kx-21N, Serial Number: A6695, as used by Archibong et al. [19].

Statistical analysis of data

Data are presented as mean ± SEM. Data were analyzed using one-way analysis of variance (ANOVA) and then followed by post hoc test (least square deviation). Data analysis was done with the help of computer software (Excel and SPSS version 17.0 for windows). P-values of less than 0.05 were considered as significant.

RESULTS

Lethality studies

Figure 1 shows that the LD_{50} value from extract of *Moringa oleifera* was 1872.22 mg/kg.

Red blood cell count result

As shown in Table 1, the RBC count (10^6 cells/mm^3) in the salt fed group (7.79±0.31) was significantly higher (P<0.05) when compared with control (6.96 ± 0.13) and *M. oleifera* (6.71±0.31) groups respectively but this was significantly (p<0.05) reduced in comparison with the salt + *M. oleifera* extract treated group (7.07± 0.12).

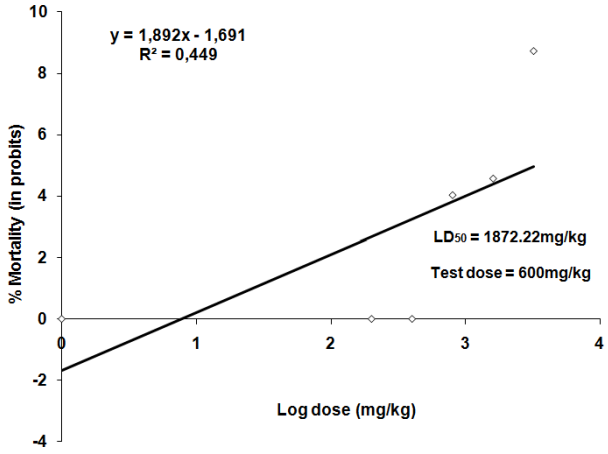

Figure 1. Lethality studies showing the effects of administering graded doses (200-3200 mg/kg i.p. mice) of *M. oleifera* extract against the percentage mortalities (converted to probits).

Hemoglobin (Hb) concentration

As shown in Table 1, there was no significant difference between the mean Hb concentration (g/dl) in the control group (12.74 ± 0.30) and the different experimental groups which are *M. oleifera* group (11.99±0.69), salt fed group (12.17±0.21) and salt + *M. oleifera* treated group (12.33±30) respectively.

Packed cell volume (PCV)

As shown in Table 1, the Packed cell volume (%) in the salt fed group (46.32±0.84) was significantly higher (P<0.05) compared with control group (42.84 ± 1.18) and *M. oleifera* group (42.23±0.58) respectively, but this was significantly (p<0.05) reduced following treatment with salt + *M. oleifera* extract (41.76±1.05).

Red blood cell distribution width (RDW-SD)

As shown in Table 1, the RDW (fl) in the salt fed group (37.21±0.49) was significantly higher (p<0.05) compared with control (35.09 ± 0.94) and *M. oleifera* extract (33.07±0.72) groups respectively but was significantly (P<0.05) lower when compared with the salt +*M. oleifera* extract treated group (32.05± 0.67).

Absolute red cell values

Mean corpuscular volume (MCV)

As shown in Table 1, there was no significant difference between the MCV (fl) in the control group (61.58 ± 1.22) and the different experimental groups which are *M. oleifera* group (63.92±8.46), salt fed group (59.78±1.66) and salt + *M. oleifera* treated group (59.25±2.12) respectively.

Mean corpuscular hemoglobin (MCH)

As shown in Table 1, the MCH (Pg) in the salt fed group (15.78±0.77) was significantly lower ($p<0.01$) compared with control (18.31 ± 0.20) and *M. oleifera* extract (17.90±0.40) groups respectively but this was significantly ($P<0.05$) reversed following treatment with the salt + *M. oleifera* extract (17.47±0.39).

Mean corpuscular hemoglobin concentration (MCHC)

As shown in Table 1, the MCHC (Pg) in the salt fed group (26.35±0.83) was significantly lower ($p<0.05$) compared with control (29.76 ± 0.36) and *M. oleifera* extract (28.33±1.38) groups respectively but this was significantly ($P<0.05$) reversed following treatment with the salt + *M. oleifera* extract (29.65±1.18).

White blood cell and differential count

As shown in Table 2, total white blood cell (WBC) count in the salt + *M. oleifera* (10.85±1.06) group was significantly higher ($p<0.05$) when compared with that of control group (7.77 ± 0.45), *M. oleifera* group (6.50±1.31), salt group (6.88±0.75), respectively. The differential cell count results are as shown in Table 1.

Platelet and platelet indices

As shown in Table 3, there was no significant difference between the mean PLT count (x1000 cells/µl) in the control group (639.67± 8.86) and the different experimental groups which are *M. oleifera* group (719.50±60.52), salt fed group (479.00±90.29) and salt + *M. oleifera* treated group (749.83±41.11), respectively. The platelet indices results are as shown in Table 3.

Table 1. Comparison of the full blood count results between the control and other groups.

	RBC (10^6cell/mm^3)	Hb	PCV (%)	MCV (fl)	MCH (Pg)	MCHC (Pg)	RDW (fl)
Control	6.96±0.13	12.74±0.30	42.84±1.18	61.58±1.22	18.31± 0.20	29.76±0.36	35.09±0.94
M. oleifera	6.71±0.37	11.99±0.69	42.23±0.58	63.92±8.46	17.90±0.40	28.33±1.38	33.07±0.72
Salt fed	7.79±0.31*	12.17±0.21	46.32±0.84*	59.78±1.66	15.78±0.77**	26.35±0.83*	37.21±0.49*
Salt + *M. oleifera*	7.07± 0.12	12.33±0.30	41.76±1.05	59.25±2.12	17.47±0.39*	29.65±1.18*	32.05±0.67

Values are represented as Mean ± SEM. *p<0.05, **p<0.01 vs control.

Table 2. Comparison of WBC and differential countresult between the control and other groups.

	WBC (10^3 cell/µl)	L	N	E	M	B
Control	7.75 ± 0.45	80.83± .60	13.67±1.56	1.50±0.43	3.50±0.43	0.50±0.22
M. oleifera	6.50±1.31	62.00±0.97***	32.50±1.20***	1.67±0.49	3.50±0.22	0.33±0.21
Salt fed	6.88±0.75	81.50±1.52	12.17±0.91	2.83±0.60	3.33±0.33	0.17±0.17
Salt + *M. oleifera*	10.85±1.06*	67.50±3.97***	27.17±3.96***	1.83±0.48	3.17±0.31	0.33±0.21

Values are represented as Mean ± SEM. **p<0.05, **p<0.01, ***p<0.001 vs control.

Table 3. Comparison of the platelet count and platelet indices result between the control and other groups.

	PLT (10^3cells/µl)	PDW (fl)	MPV (%)	P-LCR
Control	639.67±8.86	9.18±0.31	7.93 ± 0.16	10.72 ± 1.25
M. oleifera	719.50±60.52	8.67±0.28	7.57 ± 0.12*	8.83 ± 0.48
Salt fed	479.00±90.29	9.33±0.31	7.67 ± 0.08	10.12 ± 0.61
Salt + *M. oleifera*	749.83±41.11	8.97±0.54	8.07 ± 0.05*	12.43 ± 0.61*

Values are represented as Mean ± SEM. *p<0.05 vs control.

DISCUSSION

This research work was aim at investigating the potency of *Moringa oleifera* leaf extract on some hematological parameters in high salt loaded albino Wistar rats. *M. oleifera* contains numerous bioactive substances which include vitamins A, B, C, E, K, calcium ion, iron, potassium, proteins, traces of carotenoids, saponin, sistosterol, glycosides, phytate, flavonoids, kaempferol, quercetin, zeatin, alkaloids, steroids and phenolic compound. The lethality studies showed high value of LD_{50} indicating that *M. oleifera* leaf extract has very wide safety margins and hence could be relatively non-toxic. This is consistent with the observation that certain products from these leaves find therapeutic application worldwide without any known side effect.

This study revealed that the RBC count in the high salt fed group was significantly increased compared with that of the control and those groups that received extract, in consistence with recent findings [16]. This increase was due to a high degree of dehydration following high salt intake [21]. Treatment of high salt fed rats with *M. oleifera* extract reversed the increase in RBC count induced by salt loading. It is obvious that increase in RBC would invariable increase the viscosity of blood, thereby leading to an increase in blood pressure and hypertension [22]. The ability of the extract to reverse this increase points to its potential effect in managing elevated blood pressure occasioned by increase in viscosity of blood following high salt treatment.

Although the difference in hemoglobin concentration among the groups was of no statistical significance, the PCV was increased in rats that received salt relative to other experimental groups, supporting the increase in RBCs. PCV is increased following dehydration, but this effect was reversed following treatment with *M. oleifera*.

In this study the RDW-SD was significantly raised in high salt fed rats compared with the control and extract treated groups. Increase RDW is an indicator of anisocytosis, hence high salt load could cause anisocytosis which was reversed following treatment with *M. oleifera* extract.

This study revealed that there was no significant difference in the MCV in the different experimental groups. Indicating that the cell size was not adversely altered following difference treatment. Nevertheless, the slight reduction in MCV of the group that were given salt points to the tendency for microcytic anemia. Also the MCH and MCHC were significantly lowered in the group that was given salt in comparison with control and extract treated groups. Low MCH and MCHC are indications of hypochromic anemia. Showing that high salt loading can cause hypochromic anemia, but this situation was reversed following treatment with *M. oleifera* extract.

WBC count and neutrophils counts in the high salt group was significantly lower than that of control groups, but this effect was reversed following extract treatment, in agreement with findings carried out by [23, 24], which shows that *M. oleifera* extract boost the immune system. This is an indication that the extract is capable of stimulating the hemopoietic system [25].

The platelet indices results show that the platelet count was significantly decreased in the group that received salt compared with others. A decrease in platelet count could lead to excessive bleeding tendencies, purpura and leukemia which if severe may lead to death [21]. This tendency was reversed by the *M. oleifera* extract which is known to contain beta sistosterol that plays a major role in platelet formation [26]. The extract may contain thrombopoietin-like agents which are capable of stimulating the release of thrombopoietin [27] Pointing to the ability of the extract to boost platelet count even in deleterious situations like high salt loading.

The PDW-SD was found to be of no significant difference among the experimental groups indicating that there was no significant variability in size of the platelet, but the MPV and PLC-R were significantly higher in the salt treated group compared with other experimental groups. This result is of significance because MPV and P-LCR when measured have been found to be inversely related to platelet count [28]. MPV is mostly increased following platelets destruction. Increase in MPV and PLCR are implicated in the etiology of cardiovascular diseases.

CONCLUSION

In conclusion consumption of *Moringa oleifera* leaf extract ameliorates the adverse effects of salt loading on the blood cells. Therefore, the extract is recommended to pharmaceutical industries for further research and possible use in the manufacture of drugs that are necessary in management of blood pressure and other related ailments.

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other facilities for the study.

AUTHORS' CONTRIBUTIONS

This work was carried out in collaboration between all authors. ANA wrote the first draft of the manuscript, managed the literature search, carried out the feeding regimen and analysis of blood, CON designed the study and wrote the protocol while OEO performed the statistical analysis and manuscript editing. All authors read and approved the final manuscript.

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