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Instructions to Authors

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ii. Walker M, Hull A. Preterm labor and birth. In: Taeusch HW, Ballard RA, eds. Avery's Diseases of the Newborn. Philadelphia: WB Saunders, 1998: 144,153.

iii. Hagström H, Nasr P, Ekstedt M, et al. Fibrosis stage but not NASH predicts mortality and time to development of severe liver disease in biopsy-proven NAFLD. J Hepatol 2017; 67: 1265-73. doi: 10.1016/j.jhep.2017.07.027.

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Case Report

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Original Article

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Comparison of the predictive utility of Revised Trauma Score, Emergency Trauma Score, and Glasgow Coma Scale-Age-Pressure scores for emergency department mortality in multiple trauma patients

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ABSTRACT

Objective: In this study, we aimed to compare the utility of Revised Trauma Score (RTS), Glasgow Coma Scale, Age, and Systolic Blood Pressure (GAP) scores, and Emergency Trauma Score (EMTRAS) in multiple trauma patients for the prediction of mortality in the emergency department (ED).

Materials and Methods: In this observational diagnostic accuracy study, a consecutive convenience sample of all adult patients (older than 16 years) with multiple trauma (injuries confined to at least two body regions) admitted to the trauma bay of the ED during the shifts of the researchers was used. Presence of ED mortality was recorded, and RTS, EMTRAS, and GAP scores were calculated at the analysis stage of this study.

Results: The study sample included 279 multiple trauma patients. Of the 279 patients, 13 (4.7%) died in the ED. Among the 266 patients who survived to hospital admission, 3 were lost to-follow-up (foreigner patients). In the following 30 days, 28 more patients were lost, 23 in the Intensive Care Unit (ICU) (23/62, 37.1%), 4 in the wards (4/131, 3.1%), and 1 after discharge (1/73, 0.1%). The prognostic accuracies (AUC) of RTS, EMTRAS, and GAP were 0.92, 0.94, and 0.93, respectively, for ED mortality.

Conclusion: In this study, all trauma scores performed similar in the ED for the prediction of ED mortality.

Keywords: RTS, EMTRAS, GAP, Trauma, Score, Mortality

1. INTRODUCTION

In a pilot study on trauma scoring systems (TSSs) in 1981, five independent predictors of trauma outcome were determined and combined to form the Trauma Score (TS). These five predictors comprise the Glasgow Coma Scale (GCS) score, respiratory rate (RR), respiratory expansion, systolic blood pressure (SBP), and capillary refill [1]. Use of the TS resulted in reliable and accurate prediction of survival after trauma; however, evaluation of capillary refill and respiratory expansion was difficult. Therefore, few years later, the TS was revised by removing these two variables, and the Revised Trauma Score (RTS) was developed, which uses certain coefficients to give higher weight to the GCS score (Table I) [2]. The RTS was accepted by the trauma community worldwide.

Another scoring system is the GCS, Age, and SBP (GAP) scores, which is calculated with the help of each component in its name (Table II) [3]. Compared with RTS, it includes age instead of RR. It is a physiologic TSS that was developed from a multicenter study of 35,732 patients from the Japan Trauma Data Bank (JTDB) who were 16 years or older and had an Injury Severity Score (ISS) of higher than 3. The *c* statistics of GAP scores in the validation data set (0.933 for long-term mortality and 0.965 for short-term mortality) were comparable with those of the RTS (0.919 and 0.966, respectively) [3]. The GAP required fewer parameters and was applicable in the field, and its predictive utility was close to that of the RTS.

Recently, the roles of base deficit (BD), lactate, and traumatic coagulopathy have been better understood in trauma, which led to the idea of using these parameters for the estimation of prognosis. From this idea, the Emergency Trauma Score (EMTRAS) was developed, which is a scoring system that depends on readily available clinical parameters (age, prothrombin time or International Normalized Ratio (INR), and BD) (Table III) [4]. EMTRAS has an accuracy (area under the curve [AUC]) of 0.812 (95% confidence intervals [CI],

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0.795–0.829) for in-hospital mortality in its derivation and of 0.828 (95% CIs, 0.792–0.865) in a validation cohort. However, this AUC was considerably lower than that of the other scores, probably because of a missing component, that was, anatomical injury severity [5]. Smaller studies from countries other than Germany, such as Korea (AUC = 0.91; 95% CI, 0.87–0.94) [6], the Netherlands (AUC = 0.92 and 0.94) [7], and Italy (AUC = 0.80) [8], showed significant variability in the AUCs of EMTRAS.

Trauma scoring systems other than RTS, GAP, and EMTRAS are extremely hard to calculate at the bedside because of the high number of parameters included. Moreover, it is not clear how these scores compare in the emergency department (ED). Although, both EMTRAS and GAP scores may successfully predict mortality, they were not compared to each other in terms of mortality in the ED or survival to hospital admission.

Therefore, in this study, we aimed to compare the utility of RTS, EMTRAS, and GAP scores in multiple trauma patients for the prediction of mortality in the ED.

2. MATERIALS and METHODS

This observational diagnostic accuracy study was conducted, in accordance to the statement of Standards for Reporting of Diagnostic Accuracy Studies (STARD) [9], at a Level 1 trauma center with 5000 multiple trauma admissions annually, after the approval by the Institutional Ethics Committee (Approval date and number: 20.09.2013 / 02.2013.0224).

All adult patients (older than 16 years) with multiple trauma (injuries confined to at least two body regions) admitted to the trauma bay of the ED between November 10, 2013, and November 10, 2015, were defined as the source population.

All patients were managed by the attending emergency physician according to the Advanced Trauma Life Support (ATLS) guidelines [10]. During the primary survey, an initial physical examination was performed, vital signs were recorded (temperature, RR, peripheral O_2 saturation, SBP, diastolic blood pressure (DBP), and GCS score), and blood was drawn for routine analysis (venous blood gases, hemogram, coagulation panel, blood type and match, electrolytes, blood urea nitrogen, and creatinine). The location(s) (head and neck, trunk, or extremity) and etiology (blunt, penetrating, motor vehicle, fall, etc.) of the trauma were also recorded.

At this point, researchers were alerted, and all data regarding this study were collected at the bedside.

The final study population was a convenience sample consisted of patients admitted during the shifts of the researchers. Patients were excluded from the study if blood for venous gases was not drawn within the first 30 min of the admission.

The primary endpoints for outcome assessment were the mortality rate in the ED. ED mortality was defined as death by any cause while the patient was in the ED (before admitting to a ward, ICU or transfer to another facility).

Revised Trauma Scores, EMTRAS, and GAP scores were calculated according to definitions in previous studies, with the data collected during the ED admission (Tables I, II, III) [2-4].

Table I. *Calculation of the RTS score and risk stratification [2]*

Score	GCS Score	SBP (mm Hg)	RR (/min)
4	13–15	>89	10–29
3	9–12	76–89	>29
2	6–8	50–75	6–9
1	4–5	1–49	1–5
0	3	0	0

RTS: Revised trauma score,GCS: Glasgow coma scale, SBS: Systolic blood pressure, RR: Respiratory rate.

RTS is calculated by adding the weighted sum of the three scores according to the above chart = ([0.9368 × GCS score] + [0.7326 × SBP score] + [0.2908 × RR score]).

Table II. *Calculation of the GAP score and risk stratification [3]*

Parameter	Score
Age <60	3
SBP >120 mm Hg	6
SBP 60–120 mm Hg	4
SBP <60 mm Hg	0
GCS score (3–15)	3–15
Total	3–24

GAP: Glasgow coma scale (GCS)+Age+Systolic blood pressure (SBP).

Kondo et al. [3] defined the risk categories of the GAP score as follows: Scores from 3 to 10 indicate high (>50%); 11 to 18, moderate (>5% and <50%); and 19 to 24, low risk (<5%) of death up to 30 days.

Table III. *Calculation of the EMTRAS score and risk stratification [4]*

Score	Age	INR	BD	GCS
0	<40	<1.25	>-1	13–15
1	40–60	1.5–2	−1–−5	10–12
2	60–75	2.1–5	−6–−10	6–9
3	>75	>5	<−10	−5

INR:International normalised ratio, EMTRAS: Emergency TRAuma Score, BD: Base deficit, GCS: Glasgow coma scale. EMTRAS is calculated by the sum of each parameter, and a total 0–12 is obtained, indicating best to worst prognosis, respectively.

Statistical Analysis

Continuous variables were summarized as means, standard deviations, and 95% confidence intervals (CIs) or medians and interquartile ranges according to the distribution of the variable according to normality tests. Categorical variables were summarized as frequencies and percentages. Mean or median values among groups of continuous variables were compared using *t* test, analysis of variance, and Mann–Whitney *U* or

Kruskal–Wallis test. The chi-square test was used to compare categorical variables among groups. Accuracy (AUC), sensitivity, specificity, positive and negative predictive values, and positive (+LR) and negative (–LR) likelihood ratios were calculated from the contingency tables of index tests versus mortality data. The Youden J Index test was used to calculate the optimal threshold value with the highest combined sensitivity and specificity for each index test for each outcome on the Receiver Operating Curves (ROCs). In this study, the accepted Type 1 error was 5%. MedCalc Statistical Software version 18.6 (MedCalc Software bvba, Ostend, Belgium; https://www.medcalc.org; 2016) was used for all analyses.

3. RESULTS

A total of 279 multiple trauma patients were enrolled to the convenience sample of this study. The demographics, vital signs, laboratory findings, trauma scores, trauma localizations were summarized and compared according to ED mortality in Table IV. Of the 279 patients, 13 (4.7%) died in the ED. Five of the 13 patients were resuscitated as soon as they arrived in the ED. Among the 266 patients who survived to hospital admission, 3 were lost to-follow-up (foreigner patients). In the following 30 days, 28 more patients were lost, 23 in the ICU (23/62, 37.1%), 4 in the wards (4/131, 3.1%), and 1 after discharge (1/73, 0.1%). Mortality rate after hospital admission was 10.5% (n=28/263, 3 unknown). Overall 30-day mortality was 14.9% (n=41/276, 3 unknown). The mortality difference of 5.8% between before and after hospital admission was statistically not significant (4.7% vs 10.5%, 95% CI of the difference – 19.93% to 22.74%, p=0.5439).

The prognostic utility of the trauma scores is presented in Table V. Screening of other demographic factors, vital signs, and laboratory values revealed that the GCS score was the only prognostic variable, with an AUC higher than 0.90 (Table VI).

Table IV. *Demographics of the patients with multiple traumas (N = 279)*

Variable	Overall (n=279)	Survivors (n=266)	Non-Survivors (n=13)	P
Age, median (IQR), years	37 (28–51)	37 (28-50)	52 (30-71)	0.1616
Male sex, n (%)	239 (85.7)	229 (86.1)	10 (76.9)	0.3580
Occupational accident, n (%)	49 (17.6)	47 (17.7)	2 (15.4)	0.8329
Vital signs and laboratory findings, median (IQR), n=(survivor/non-survivor)				
GCS score	15 (14–15)	15 (14-15)	3 (3-6)	<0.0001
SBP, mmHg (n=264/13)	123 (110–130)	124 (110-130)	65 (0-108)	<0.0001
DBP, mmHg (n=263/13)	78 (67–89)	78 (68-89)	33 (0-58)	<0.0001
RR, /min (n=264/13)	16 (15–20)	16 (15-20)	6 (0-14)	0.0012
Base excess, mEq/L (n=192/11)	1.2 (0.8-4.7)	1.2 (-0.9-4.0)	12.9 (8.5-19.0)	<0.0001
INR (n=260/11)	1.13 (1.05–1.22)	1.12 (1.05-1.21)	1.46 (1.30-1.56)	<0.0001
Lactate, mg/dL (n=194/13)	2.6 (1.9–3.9)	2.5 (1.9-3.6)	10.2 (5.2-12.1)	<0.0001
pCO2, mm Hg (n=193/13)	43.7 (36.9–49.9)	68.3 (44.9-85.7)	43.1 (36.9-48.3)	0.0034
Trauma scores, median (IQR)				
RTS	7.841 (7.550–7.841)	7.841 (7.841-7.841)	3.512 (0-4.624)	<0.0001
EMTRAS	2 (1–3)	1 (0-2)	6 (5-7.25)	<0.0001
GAP	22 (21–24)	22 (21-24)	9 (5.25-14.0)	<0.0001
Mortality after ED, n/N (%)				
Mortality in surgery/wards	4/131 (3.1)			
Mortality in ICU	23/62 (37.1)			
Mortality after discharge	1/73 (0.1)			
Mortality total, n/N (%)	41/276 (14.9)			
Location, n (%)				
Other (soft tissue, peripheral nerves, vessels)	171 (61.3)	161 (60.5)	10 (76.9)	0.2368
Thorax and mediastinum	139 (49.8)	132 (49.6)	7 (53.8)	0.7667
Head–neck–face	117 (41.9)	108 (40.6)	9 (69.2)	0.0415
Extremities	97 (34.8)	89 (33.5)	8 (61.5)	0.0383
Cranium and central nervous system	93 (33.3)	82 (30.8)	11 (84.6)	0.0001
Spine	73 (26.2)	71 (26.7)	2 (15.4)	0.3660
Trunk/abdomen	62 (22.2)	54 (20.3)	8 (61.5)	0.0005
Pelvis	49 (17.6)	44 (16.5)	5 (38.5)	0.0429

Three patients of foreign origin were excluded from the study because they were lost to follow-up and their data could not be attained. IQR: Interquartile range, GCS: Glasgow coma sclae, SBP: Systolic blood pressure, DBP: Diastolic blood pressure, RR: Respiratory rate, INR:International normalised ratio, RTS: Revised trauma score, EMTRAS: Emergency Trauma score, GAP: Glasgow coma scale+Age+Blood pressure, ICU:Intensive care unit.

Table V. *Utility of the RTS, EMTRAS, and GAP for ED mortality*

	RTS (n = 277)	EMTRAS (n = 197)	GAP (n = 277)
AUC (95% CI)	0.92 (0.88–0.95)	0.94 (0.90–0.97)	0.93 (0.90–0.96)
P	**<0.001**	**<0.001**	**<0.001**
Sensitivity (95% CI)	92.3 (64.0–99.8)	100.0 (69.2–100.0)	92.3 (64.0–99.8)
Specificity (95% CI)	87.5 (82.9–91.2)	80.8 (74.4–86.1)	86.4 (81.6–90.3)
+LR (95% CI)	7.38 (5.2–10.5)	5.19 (3.9–7.0)	6.77 (4.8–9.5)
–LR (95% CI)	0.088 (0.01–0.60)	0.0	0.089 (0.01–0.60)

RTS: Revised Trauma Score, EMTRAS: Emergency Trauma Score, GAP: Glasgow coma scale+Age+Blood pressure, AUC: Area under curve, LR: Likelihood ratios, CI: Confidence interval. (Three patients of foreign origin were excluded from the study because they were lost to follow-up and their data could not be attained.)

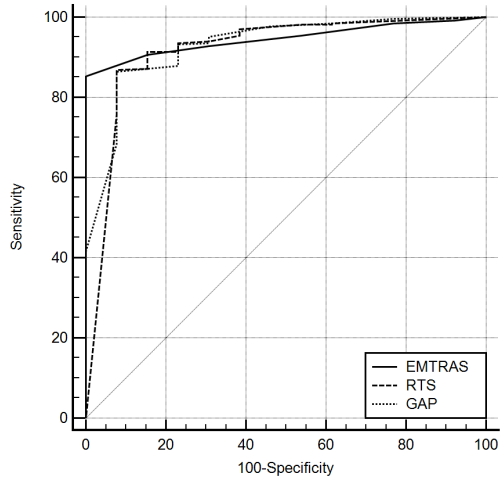

Figure 1. *Comparison of the ROC curves of trauma scores regarding ED mortality*

Table VI. *Prognostic utility of other markers for mortality prediction*

Variables	ED Mortality		
	N	AUC (95% CI)	P
Age	279	0.615 (0.555–0.672)	0.2221
SBP	277	0.778 (0.724–0.826)	**0.0062**
DBP	275	0.826 (0.775–0.869)	**0.0005**
RR	277	0.764 (0.710–0.813)	**0.0197**
INR	271	0.857 (0.810–0.897)	**<0.0001**
BE	203	0.881 (0.829–0.922)	**<0.0001**
Lactate	207	0.893 (0.842–0.931)	**<0.0001**
pCO2	206	0.743 (0.677–0.801)	**0.0271**
PTT	271	0.847 (0.799–0.888)	**0.0001**
GCS	279	0.903 (0.862–0.935)	**<0.0001**

The statistically significant P values are denoted in bold. AUC: Area under the curve, SBP: Systolic blood pressure, DBP: Diastolic blood pressure, RR: Respiratory rate, INR:International normalised ratio, BE: Base excess, pCO2: Partial pressure of carbon dioxide, PTT: Partial thromboplastin time, GCS: Glasgow coma scale

The pairwise comparison of the AUCs of the ROCs of scores did not reveal any significant difference (Figure 1).

The ED mortality rates were 9 of 21 (42.9%), 3 of 28 (10.7%), and 1 of 228 (0.4%) for high (3–10), moderate (11–18), and low (19–24) GAP risk classes. +LR to rule in ED mortality in the high-risk group was 15.2 (95% CI, 7.9–29.5), and –LR to rule out ED mortality in the low-risk group was 0.09 (95% CI, 0.01–0.59).

4. DISCUSSION

In this study, we aimed to compare the utilities of RTS, EMTRAS, and GAP scores in predicting ED mortality in multiple trauma patients, and found that they were highly accurate (AUCs: 0.92, 0.94, and 0.93, respectively, Table V), without any significant differences between each other.

The prognostic utility of the RTS was evaluated in countries with lower resources: In 2014, Ahun et al., reported the AUC of RTS for ED mortality as 0.727 (P= 0.012), in their study in Turkey [11]. In a study from Malaysia, the AUC of RTS for in-hospital mortality was reported to be 0.80 [12]. In another study from Iran, the AUC of RTS for the prediction of in-hospital mortality was reported to be 0.86 (95% CI, 0.82–0.90) [13]. A recent study from Korea reported the AUC of RTS to be 0.92 (95% CI, 0.91–0.93) [14]. In our study, we also found the AUC of RTS for ED mortality as 0.92. The prognostic utility of RTS seems to be high and similar in middle-income countries, which have a more limited healthcare system with a higher mortality rate in trauma.

Research on the utility of the EMTRAS score is insufficient. We were able to locate only four studies that reported on the accuracy of EMTRAS for mortality in the literature. The first study to propose EMTRAS reported the AUCs for in-hospital mortality as 0.81 (95% CI, 0.80–0.83) and 0.83 (95% CI, 0.79–0.87) in derivation and validation cohorts, respectively [4]. In a separate validation study, Mangini et al., published the preliminary results of a single-center study of 150 patients and reported an AUC of 0.81 (95% CI, 0.74–0.87) [15]. In a retrospective, single-center study from Korea by Park et al., the predictive values of EMTRAS, RTS, ISS, and Rapid Emergency Medicine Score (REMS) were compared regarding in-hospital mortality in 6905 trauma patients and reported an accuracy of 0.96 for EMTRAS [16]. The last study was a prospective, observational study from Tunisia by Hamed et al. [17]. They evaluated the prognostic performance of trauma scores in terms of mortality at 30th day

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in severe trauma patients and found the AUC of EMTRAS for 30th day mortality as 0.789, and defined an EMTRAS score of 3 or above as an independent predictor of mortality (adjusted OR 1.80, 95% CI [1.05-3.08], p = 0.0033). We found the AUC of EMTRAS for ED mortality as 0.94, and our results are quite similar to research conducted by Park et al. [16].

In 2011, Kondo et al., developed GAP score and calculated the threshold values for low-, moderate – and high-risk categories [3]. They stated that the *c* statistics for the GAP scores (0.93 for long-term mortality and 0.97 for short-term mortality) were better than those for the other trauma scores. Later, Ahun et al. [11], reported that the AUCs of the GAP score for short – and long-term mortality as 0.910 and 0.904, respectively, which were similar to those of the Kondo et al. [3], and Hamed et al. [17], reported an AUC value for GAP as 0.811 for mortality at 30th day, leading score among the compared scores in their study, and defined a GAP score below 20 as an independent predictor of mortality (adjusted OR 1.92, 95% CI [1.268-2.92], p = 0.002). In this study, we found the AUC of GAP as 0.93 for ED mortality, and confirmed the previous studies. A pairwise comparison of the ROC values of trauma scores revealed no statistically significant difference in our study.

Limitations

The limitations of this study should be acknowledged. First, the consecutive sampling of the patient population when the researcher of this study was present in the ED may have created a sampling bias. However, based on the demographics of the study population, it was deemed that this was a good sample of our patient population. Second, patients who died at the scene were eventually excluded, which may have caused, in part, a spectrum bias. However, because this is a study on prognostic scores, the severity of trauma of all patients included in the study is given in detail, which decreases the effect of this bias.

Conclusion

In this study, all trauma scores performed similar in the ED for the prediction of ED mortality.

Acknowledgment

We would like to thank the Department of Radiology and Department of Biochemistry for their hard work while preparing the official reports

Compliance with Ethical Standards

Ethical Approval: The study protocol was approved by the Local Ethics Committee (Approval date and number: 20.09.2013 / 02.2013.0224).

Financial Support: The authors have no relevant financial information to disclose.

Conflict of Interest: The authors have no potential conflicts to disclose.

Authors' contributions

Initials of the contributing authors were listed in brackets after the relevant parts of the research: Literature search (MES, HA), study design (MES, HA, OO, AD), legislative applications (MES, HA), data collection (MES), supervision and quality control (HA, OO, ADA), statistical advice (HA, OO, AD), statistical data analysis (HA), data interpretation (MES, HA), drafting the manuscript (MES, HA). All authors were involved in the writing and critical revision of the manuscript and approved the final version. ME and HA took responsibility for the paper as a whole.

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Examination of the apoptotic effects of betulinic acid on renal cancer cell lines

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ABSTRACT

Objective: Renal cancers are highly resistant to standard hormonal therapy, radiotherapy, and chemotherapy, and the survival rates are extremely low. Betulinic acid is a pentasilic triterpenoid saponin of lupine type obtained from various natural plants, especially from the shell of *Betula* plant. Betulinic acid was shown both in *in vivo*, and *in vitro to* have the ability to induce apoptotic pathways causing no toxicity for normal cells, and also has immunomodulatory effects. The aim of the present project is to investigate the anticancer effects of betulinic acid on CAKI-2 (ATCC® HTB-47™; clear cell renal carcinoma), ACHN (ATCC® CRL-1611™; renal cell adenocarcinoma) and MRC-5 (ATCC® CCL-171™: normal lung fibroblast) cell lines.

Materials and Methods: The dose, and time-dependent cell viability was determined using the WST-1 test first in cell lines, and then apoptotic activity was determined with Annexin-V, apoptosis related nucleosomal enrichment factor levels, and Caspase 3 / BCA activity.

Results: Betulinic acid reduced the CAKI-2, and ACHN cell viability in dose, and time-dependent manner inducing the apoptotic pathway.

Conclusion: Researchers in the present study concluded in accordance with the results of Annexin-V, apoptosis-associated nucleosomal enrichment factor levels and Caspase 3 / BCA activity that betulinic acid triggered the apoptosis in both renal cancer cell lines, especially by the Caspase 3 activity.

Keywords: Renal cancer, Betulinic acid, Apoptosis, ACHN, CAKI-2

1. INTRODUCTION

Triterpenoids are the metabolites of isopentenyl pyrophosphate oligomers, and constitute the largest group of natural plant products with over 20.000 known members [1]. Triterpenoids have recently received much attention owing to their various promising biological, and pharmaceutical properties, including anti-tumor, antiviral, antifungal, anti-inflammatory, and other activities [2].

Betulinic acid is a pentacyclic triterpeniod saponin of the lupane type obtained from various natural plants, in particular from the shell of the *Betula* plant. Betulinic acid has recently been shown to have a pronounced anti-tumoral effect in melanoma cells, and in various solid tumors, including glioblastoma, lung carcinoma, colorectal carcinoma, breast carcinoma, and prostate carcinoma [3]. Betulinic acid is different than the other known anti-tumor drugs such as doxorubicin. It is a member of a new class of potential anticancer drugs that have the ability to induce apoptosis by directly affecting the mitochondria [4-6]. Betulinic acid therapy inhibits Nuclear Factor kappa-light-chain-enhancer of activated B cells (NF-kB) activation by different stimulants in colon cancer, and lung adenocarcinoma cells. These properties of betulinic acid can be used as a starting point to prevent cancer [7]. The cytotoxicity, and anti-tumor properties of betulinic acid and its derivatives have been investigated using xenograft mouse models, as well as in many human tumor cell lines [8-10]. The efficacy of Menyanthes trifoliate-L derivatives including betulinic acid on stage IV glioblastoma cell line was investigated in a recent study, and was shown to have an effect on Bcl-2-associated X protein (Bax), B-cell lymphoma 2 (Bcl-2), caspase-3 (Cas-3), and tumor protein 53 (p53) gene expression levels, and decreased the mitochondrial membrane potential [11]. The combination of various chemotherapy agents (i.e. doxorubicin, etoposide, taxol, cisplatin, actinomycin

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D, mitramycin A, or vincristine) with betulinic acid induces apoptosis, and inhibits the clonogenic structure of many tumor cells [12]. In cancer treatment, all these metabolic pathways, especially the targeted ones, are the important goals in the treatment process. The Lamin 1 gene was suggested as a new therapeutic target for betulinic acid in a microarray study on pancreatic cancer [13]. The anti-cancer activity of betulinic acid was investigated in 60 drug-resistant cell lines in a single array study including ACHN and CAKI-1, one of the kidney cancer cell lines. Saeed et al., reported that betulinic acid exhibited extremely high cytotoxicity against multidrug resistant (MDR) cell lines, and the increase in MDR activity experimentally did not alter the efficacy of betulinic acid, and more importantly, the cytotoxic activity of betulinic acid was demonstrated to occur through different mechanisms. However, there was no detailed examination of the pathways where betulinic acid was effective in kidney cancer cell lines in that study [14].

It was clear in the literature that the studies on the effectiveness of betulinic acid and cancer are still popular at present, and betulinic acid is advancing confidently to be a potential drug for anti-cancer therapy. In the present study, we aimed to demonstrate the possible apoptotic efficacy of betulinic acid on renal cancer cell lines using different methods.

2. MATERIAL and METHODS

Cell Culture

CAKI-2 (ATCC® HTB-47™) clear cell renal carcinoma, ACHN (ATCC® CRL-1611™) renal cell adenocarcinoma isolated from the metastatic site, and a healthy cell line were prepared by growing in the cellular culture medium MRC-5 (ATCC® CCL-171™). The media for cell lines were McCoy's medium for CAKI-2, Eagle's Minimum Essential Medium (EMEM) for ACHN, and Dulbeco's Minimum Essential Medium (DMEM) for MRC-5. All media were prepared with the suitable concentration of fetal bovine serum (containing 10% FBS), and penicillin/streptomycin as antibiotics (containing 1%). All cells were incubated, and cultured at 37°C in 5% CO_2. The medium was refreshed in 2-day intervals until the cells reached to adequate count. After having the adequate confluency count, trypsin application was performed to remove cells from the flask, and then the basic cellular culture techniques were performed respectively. These experimental procedures were regularly repeated until the end of the project, approximately one year.

WST-1 Cytotoxicity test

Betulinic acid was administered at an increasing dose (1-100 uM), and in increasing time intervals (24th, 48th, and 72nd hours). In this context, WST-1 cytotoxicity test reached the optimization stage. The experiments were repeatedly performed in order to identify the appropriate dose, and the time.

The administered doses were: 1 uM, 2.5 uM, 5 uM, 7.5 uM, 10 uM, 25 uM, and 50 uM.

The applied time points were: 24th, 48th, and 72nd hours.

Cytotoxicity test was performed for each dose, and each time point.

CAKI-2, ACHN, and MRC-5 cells were seeded in 96 well plates at 10.000 cells for each well. After the application of betulinic acid, 10 µL of WST-1 solution was added to each well at the end of the incubations, and after 4 hours of incubation at 37 °C, the absorbance density values of 96-well plates were measured at 440 nm in the enzyme-linked immunoabsorbant assay (ELISA) plate reader to determine the cell viability. Following the optimization of the cytotoxic analysis, the dose selection was done after administration of betulinic acid at increasing dose, and durations (1-100 uM; 24th, 48th, and 72nd hours).

Annexin-V

Flow cytometric Annexin-V apoptosis/necrosis analysis was performed for the quantitative determination of the dose, and time-dependent triggered apoptosis, and necrosis. Annexin-V was conjugated with fluorescein isothiocyanate (FITC) that was used in apoptosis analysis forms Annexin-V lectin, and it binds to phosphatidyl serine phospholipid located on the outer surface of the cell membrane of apoptotic cells. In combination with the 7-AAD-DNA-staining, dye exclusion of vital cells allows a discrimination between apoptotic, and necrotic cells. Cells treated in 24-well dishes were centrifuged, washed with HBSS−/− (GIBCO, Germany) , and stained with 150 µL of buffer containing 5 µL Annexin V-FITC and 1.5 µM 7-AAD at 37 °C. After 10 min, an additional 500 µL of ice-cold buffer was added by the time the cells were placed on ice. After centrifugation, the cells were suspended in 250 µL buffer and were immediately analysed by flow cytometry using the green-collecting fluorescent channels FL-1 for Annexin V-FITC, and FL-3 for 7-AAD, as described above. Quadrant separation in the fluorescent channels FL-1 (Annexin V-FITC) versus FL-3 (7-AAD) represents the events of necrotic, and apoptotic cells.

Measurement of Apoptosis Level with Cell Death Detection Kit (Roche) in the Cell Lines

Cell death detection kit is a photometric enzyme immunoassay used for the in vitro determination of cytoplasmic histone-associated DNA fragments after induced cell death. Cell death levels were determined by the ELISA method in accordance with the kit instructions (Roche, USA).

Analysis of Caspase 3 / BCA Activity

Caspase activity measurement of betulinic acid-induced dose, and time-dependent apoptosis in CAKI-2, ACHN, and MRC-5 cells was performed with commercially available Caspase 3 colorimetric kit (BioVision Research Products,USA). Briefly, apoptosis-induced cells, and were collected by centrifugation at 1000 rpm for 10 min. Cells were lysed by adding 50 µL of chilled cell lysis buffer and

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incubated on ice for 10 min before centrifugation at 10 000 g for 1 min. Supernatants were transferred to new Eppendorf tubes, and the reaction mixture was prepared in 96-well plates by adding 50 µL of reaction buffer, 50 µL of sample, and 5 µL of DEVD-pNA substrate, and incubated at 37°C in CO_2 incubator. After incubation, the plate was read using an ELISA reader at 405 nm wavelengths.

Statistical Analysis

The statistical analysis of this study was performed using the Statistical Package for the Social Sciences (SPSS) Statistics 21.0 package program, and the statistical significance limit was accepted as P <0.05.

3. RESULTS

WST-1 Cytotoxicity test

CAKI-2, ACHN, and MRC-5 cells were treated with betulinic acid to calculate the percentage of viability, and the dose was determined. According to the cytotoxicity results, 25 uM and 50 uM betulinic acid concentrations and 24 hour incubation time were selected to continue apoptotic detection methods on the cell lines, and the experiments were continued with the selected dose, and time. In the CAKI-2 cell line, there was a 42.6% decrease in cell viability for 24 hours, and 25 uM dose of betulinic acid; while 84.3% reduction was detected for 24 hours at a dose of 50 uM. Cell viability in the ACHN cell line decreased by 53.5% after administration of 25uM betulinic acid for 24 hours; while 70.6% reduction was detected for 24 hours at a dose of 50 uM (p<0.05), there was no considerable effect of betulinic acid on viability in the healthy cell line MRC-5. The effects of betulinic acid on cell viability have been demonstrated in Figures 1, 2 and 3.

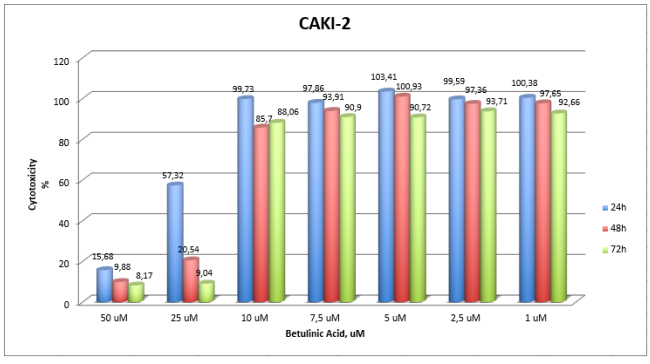

Figure 1. *Effects of betulinic acid on cell cytotoxicity for CAKI-2*

(± SD 50uM;24h:15.68±2.21, 48h:9.88±1.86 72h:8.17±1.45; 25uM; 24h:57.32±11.25, 48h:20.54±4.28, 72h:9.04±2.51; 10uM; 24h:99.73±32.69, 48h: 85.7±24.78, 72h:88.06±32.78; 7.5uM; 24h:97.86±33.62, 48h:93.91±36.88, 72h:90.9±29.45; 5uM; 24h:103.41±46.44, 48h:100.93±41.02, 72h:90.72±40.23; 2.5uM; 24h:99.59±20.11, 48h:87.36±23.56, 72h:93.71±30.01; 1uM; 24h:100.38±24.12, 48h:97.65±24.56, 72h:92.66±18.71)

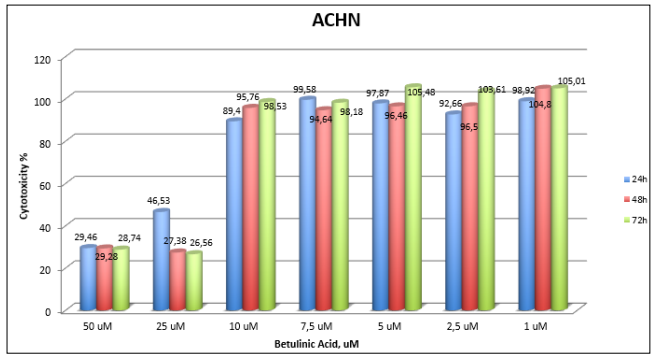

Figure 2. *Effects of betulinic acid on cell cytotoxicity for ACHN*

(± SD 50uM; 24h: 29.46±2.29, 48h: 29.28±3.86, 72h:28.74±1.95; 25uM; 24h:46.53±10.25, 48h:27.20±4.08, 72h:33.56±3.91; 10uM; 24h:89.40±14.29, 48h: 95.76±20.73, 72h:98.53±28.18; 7.5uM; 24h:99.58±33.20, 48h:94.64±30.88, 72h:98.18±27.44; 5uM; 24h:97.87±36.44, 48h: 96.46±40.00, 72h:105.48±41.33; 2.5uM; 24h:92.66±25.10, 48h:96.5±24.06, 72h:103.61±33.40; 1uM; 24h:98.92±26.85, 48h:104.80±31.36, 72h:105.01±28.14)

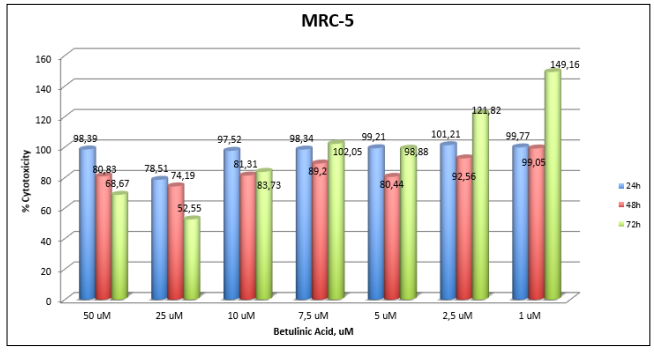

Figure 3. *Effects of betulinic acid on cell cytotoxicity for MRC-5*

(± SD 50uM; 24h: 98.39±11.29, 48h: 80.83±9.92, 72h:88.67±21.90; 25uM; 24h:78.51±19.28, 48h:74.19±22.28, 72h:52.55±3.68; 10uM; 24h:97.52±26.03, 48h:81.31±18.96, 72h:83.73±20.27; 7.5uM; 24h:98.34±30.10, 48h:89.20±28.11, 72h:102.05±30.47; 5uM; 24h:99.21±27.94, 48h:80.44±19.47, 72h:98.88±30.74; 2.5uM; 24h:101.21±30.10, 48h:92.56±24.55, 72h:121.82±34.81; 1uM; 24h:99.77±19.95, 48h:99.05±31.41, 72h:149.16±35.62)

Flow cytometric Annexin-V apoptosis/necrosis and Caspase-3/BCA, and apoptosis-related nucleosomal enrichment factor analyses were performed in order to determine the amount of apoptosis-based cell death after administration of betulinic acid at 25 uM, and 50 uM concentrations for 24 hours in the CAKI-2, and ACHN cell lines.

Annexin-V

We performed flow cytometric Annexin-V apoptosis / necrosis analysis for quantitative determination of apoptosis, and necrosis induced by dose, and time dependent concentrations of betulinic acid at 25 uM, and 50 uM doses, and at incubation time of 24 hours. Apoptotic cell death was observed in the ACHN cell line at a rate of 22.66% after the administration of 50 uM of betulinic acid, while this rate was 14.25% in the CAKI-2 cell line. We found that administration of 25 uM, and 50 uM betulinic acid had no effect on apoptosis in healthy MRC-5 cell line. The

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effects on the cell lines have been demonstrated in Figures 4, 5, and 6.

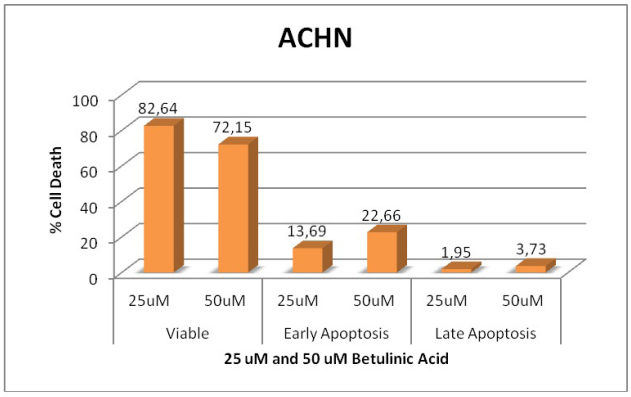

Figure 4. *Annexin-V results for ACHN*

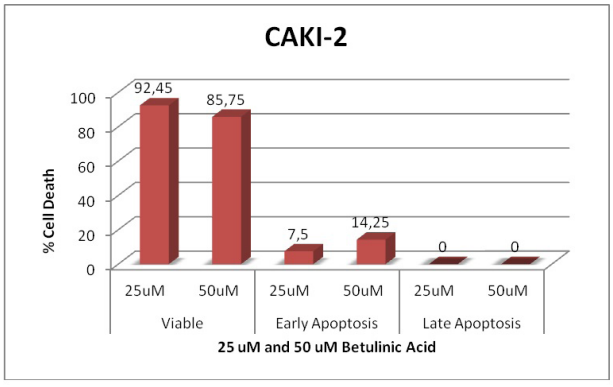

Figure 5. *Annexin-V results for CAKI-2*

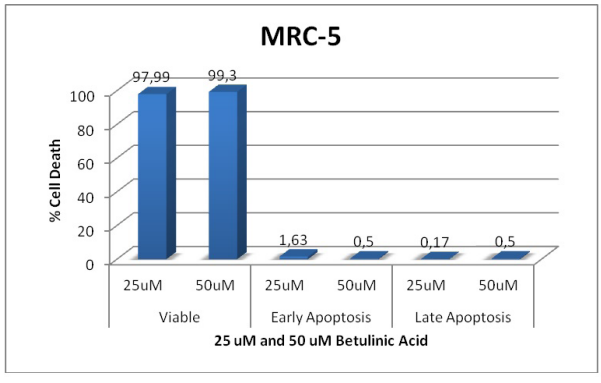

Figure 6. *Annexin-V results for MRC-5*

Measurement of Apoptosis Level in Cell Lines Treated with Betulinic Acid by the Determination of the Effect on Nucleosomal Enrichment Factor

To determine the apoptotic activity of betulinic acid on CAKI-2, ACHN, and MRC-5, the cells were treated with betulinic acid at selected dose, and time, and changes were detected for the nucleosomal enrichment factor. Accordingly, as a result of 25 uM betulinic acid application, enrichment factor increased 2.33 fold in the CAKI-2 cell line and 10.32 fold in the ACHN cell line than in MRC-5. After administration of 50 uM betulinic acid, 2.85 fold increase in the CAKI-2 cell line, and a 2.61-fold increase in the ACHN cell line were detected (Figure 7).

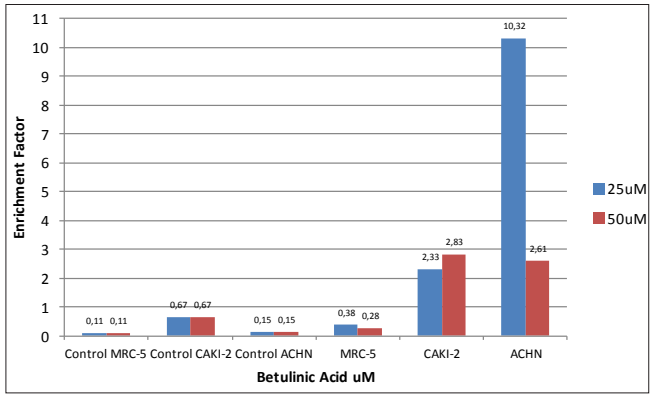

Figure 7. *Effects of betulinic acid on apoptosis related nucleosomal enrichment factors*

Caspase 3 / BCA Activity Results

The measurement of betulinic acid induced dose, and time dependent apoptosis in CAKI-2, ACHN, and MRC-5 cells by Caspase 3/BCA activity method showed that Caspase 3 activity increased by 1.4-fold as a result of 25 uM betulinic acid administration and a 1.7-fold increase occurred by 50 uM administration in the CAKI-2 cell line. In the ACHN cell line, Caspase 3 activity increased 1.14-fold after 25 uM administration, and 1.6-fold after 50 uM administraion ($P < 0.05$) (Figure 8).

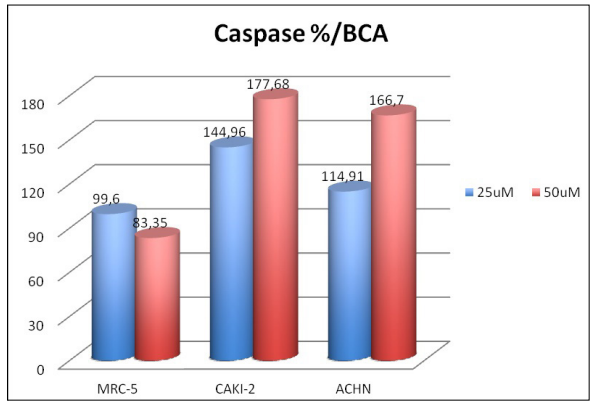

Figure 8. *Effects of betulinic acid on caspase-3 activity in cell lines*

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4. DISCUSSION

Renal cancers are highly resistant to standard hormonal therapy, radiotherapy, and chemotherapy. The average five-year survival rate is 53% in non-metastatic disease, and 8% in metastatic renal cancers. In the last 20 years, angiogenetic factors such as vascular endothelial growth factor (VEGF), platelet derived growth factor (PDGF), and mammalian target of rapamycin (mTOR) have been the main molecules for targeted treatment studies in renal cancers. Tyrosine kinase inhibitors (sunitinib, axitinib.), and mTOR inhibitors (everolimus, temsirolimus) are the drugs developed for this purpose. However, resistance against these drugs generally occurs within the first year.

Betulinic acid has potential cytotoxic effects against different cancer cells. In this experimental study, we aimed to determine the anticancer efficacy of betulinic acid in renal cancers. Therefore, Annexin-V, apoptosis-associated nuclear enrichment factor levels, and Caspase 3/BCA activity were measured following the determination of viability percentages in CAKI-2, ACHN renal cancer cell lines and the healthy cell line MRC-5 by selecting 25 uM, and 50 uM dose and 24-hour administration.

Zeng et al., showed in their study that betulinic acid triggered apoptosis via caspase-3 pathway in colorectal cancer cell lines, decreased the MMP gene expression level, and increased the TIMP-2 gene expression level. Thus, they suggested that Betulinic acid could be effective on cell migration, and metastasis and that it was a potential anti-cancer drug [15]. Yang et al. studied the anti-cancer activity of betulinic acid on renal cancer lines using the *in vivo,* and *in vitro* methods. Betulinic acid was shown to have increased the proliferation in the 786-O (human renal cell adenocarcinoma cell line) and ACHN cell lines, induced apoptosis and suppressed cell migration, and invasion [16]. Our study findings are consistent with Yang et al study in that betulinic acid induces apoptosis in renal cancer cell lines. Wang et al., showed in a study of breast cancer that the cells treated with paclitaxel-betulinic acid hybrid had higher rates of increase in apoptosis, and had less cell migration compared to cells exposed to only paclitaxel. Thus, they reported that betulinic acid positively increased the effectiveness of paclitaxel, an anticancer drug [17]. Gao et al., reported in their study on breast cancer cell lines that the typical morphological features of apoptosis occurred after betulinic acid treatment, which induced the apoptosis [18].

The present study confirmed that betulinic acid was able to promote apoptosis in renal cell carcinoma cells. Our results showed that the ratio of apoptotic cell death in ACHN, and CAKI-2 cell lines were increased compared to the numbers in MRC-5 healthy cell line. In addition, we observed that the nuclear enrichment factor levels as an indicator of apoptotic cells in ACHN, and CAKI-2 cell lines were higher compared with the MRC-5 healthy cell line, and control cells. Caspase 3/BCA activity as a marker of apoptosis has also increased in ACHN, and CAKI-2 cell lines compared to the numbers in MRC-5.

In conclusion, we found with the results of Annexin-V, apoptosis-related nucleosomal enrichment factor levels measurement and Caspase 3/BCA activity that betulinic acid triggered apoptosis in both renal cancer cell lines, particularly through the caspase 3 activity.

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Compliance with Ethical Standards

Ethical approval: According to the Institutional Ethical Committee this study did not require ethics approval as it was conducted on cell lines and the data did not contain patient-specific information. (Document number: 1566/23 12 2019)

Conflict of Interest: All the authors have declared no competing interest.

Authors' Contributions: AE: writing and supervisor; ESI: writing and cell culture procedures; BE, MNA and GK, : Cell culture procedures; BC: Statistical analysis

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Evaluation of the awareness of childhood cancers by general practitioners, family physicians and pediatricians

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ABSTRACT

Objective: Although, childhood cancer was considered as a deadly disease in the past, now it is considered as a life-threatening chronic disease if diagnosed early. The present study evaluates how much the symptoms of childhood cancers are recognised by the physicians in Istanbul, Turkey.

Materials and Methods: The objective of the study and the questionnaire form was explained to participants and they were asked whether they would like to take part in the study. The questionnaire composed of two sections: 1. Investigated the sociodemographic characteristics of the physicians and 2. Investigated the knowledge of the physicians on childhood cancers (leukaemia, solid tumour, common questions). The physicians were given 2 points for every correct answer; wrong answers and answers such as "I have no idea" were scored 0 points. Based on the correct answers given by all participants, the medians for every question group were calculated. The measure of success was 75 percentile, which was calculated for each question group considering these median values.

Results: Examining the percentages of the correct answers to the questions, the best known were the leukaemia questions (74.6%) and the less known were the solid tumour questions (57.1%). The physicians younger than 33 years, which was the mean age, gave better answers to the leukaemia questions and the common questions. When the academic titles of participants were grouped as pediatricians and other physicians, the leukaemia, solid tumour, common group and total points showed statistically high significant differences.

Conclusion: Our study has revealed the need of our country for training programs aimed at increasing the awareness of general practitioners working in primary health care institutions, as these institutions are the first reference centers for the pediatric patient population. Childhood cancer awareness must be improved by implementing training programs which in turn will lead to early diagnosis and referral to an appropriate specialist.

Keywords: Childhood cancer, Recognition, Symptom, Diagnosis

1. INTRODUCTION

Although, childhood cancer was regarded as fatal disease in the past, today it is accepted as a chronic disease that endangers life if diagnosed early [1,2]. Childhood cancer is generally in the lower ranks in differential diagnosis because it is less common than other childhood diseases and its symptoms are generally not specific. Therefore, there are delays in the diagnosis of cancer in childhood [3,4]. Considering that the treatment success is higher in children, the importance of early diagnosis and treatment can be better appreciated. The greatest responsibility at this stage is borne by the general practitioners and the pediatricians who first see the patient. The relationship between the start of the symptoms and the diagnosis time has been frequently studied up to now [5]. However, studies on the awareness of cancer symptomatology of pediatricians and especially primary care physicians are very few, almost none.

The present study aims to evaluate the awareness of pediatricians and physicians of other fields, particularly general practitioners, concerning the symptoms and diagnosis of childhood cancers.

2. MATERIALS and METHOD

The present study was conducted by the Pediatric Haematology and Oncology Division of the School of Medicine, Marmara

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University, Istanbul. The permit necessary to conduct the study was given by the Istanbul Provincial Health Directorate. The study was approved by the Ethical Committee of School of Medicine, Marmara University (Approval number: B.30.2. MAR.0.01.02/AEK/). The objective of the study and the two-page questionnaire form (Annex1) was explained to the family physicians, general practitioners, pediatricians and surgeons who might confront cancer working in all community health centres, state hospitals, training and research hospitals, university hospitals and private hospitals and they were asked whether they wanted to take part in the study. The questionnaire composed of two sections: 1.Investigated the sociodemographic particulars of the physicians participating in the study and 2. Investigated their awareness on childhood cancers (leukaemia, solid tumour, common questions concerning childhood cancers) (Annex 1). A total of 297 physicians participated in the study and completely filled in the questionnaire distributed at their work center. In the questionnaire, the physicians were given 2 points for every correct answer; wrong answers and answers such as "I have no idea" were scored 0 points. Based on the correct answers given by all participants, the medians for every question group were calculated. The measure of success was 75 percentile, which was calculated for each question group considering these median values. Above this value is accepted as successful in all question groups.

Statistical Analysis

The data were analysed using the program Statistical Package for the Social Sciences (SPSS) for Windows 19. 0. The answers to the first section questions, which investigated the sociodemographic characteristics and the answers to the second section questions were compared using appropriate statistical tests. The Mann-Whitney U test was used for comparing the parameters that have no normal distribution in quantitative data and the Kruskal Wallis test was used for comparing more than two groups. Significance level in all tests were taken as $P< 0.05$.

3. RESULTS

A total of 297 physicians participated in our study. Of these, 148 (49.8%) were female and 149 (50. 2%) male. Of the participating physicians, 126 (42.4%) were pediatricians, 67 (22.9%) were general practitioners, 64 (21.2%) were internal medicine physicians, 22 (7.7%) were surgeons and 18 (5.8%) were family physicians (Table I).

Table I. *The number of participants according to academic degree*

	N(%)
Female/Male	148 (49.8%)/149(50.2%)
Pediatricians	126 (42.4%)
General practitioners	67 (22.9%)
Internal medicine physicians	64 (21.2%)
Pediatric surgeons	22 (7.7%)
Family physicians	18 (5.8%)

It was found that 90 (30.3%) participating physicians had cancer in the family history and 140 (47.1%) of the participating physicians was diagnosed with cancer at least once in the past, 133 (45%) followed a child with cancer at least once in the past.

In the present study, the first section of the questionnaire included 11 questions on sociodemographic characteristics. The second section of the questionnaire form had 50 questions concerning characteristics of childhood cancers. Of these questions, 18% were about leukaemia, 48% were about solid tumours and 34% were common questions. Examining the percentages of the correct answers to the questions, the best known were the leukaemia questions (74.6%) and the least known were the solid tumour questions (57.1%). The number of correct answers for all questions is given in Annex I.

Tables II and III give the rankings of the first 5 questions that were correctly answered most frequently and that incorrectly answered most frequently and the numbers and percentages of correct answers. No statistical difference was observed when comparing total scores with respect to the gender of the participants (Mann-Whitney U test, p=0.441).

Table II. *The correct answers most frequently given by the participants*

Questions	Group	Correct answers (N) (%)
Sudden weight loss may be the first sign of cancer in a child	Leukemia group	262 (88.2%)
Sudden developing weakness – fatigue can be a sign of cancer in a child	Leukemia group	260 (87.5%)
No further investigation is needed in a child with iris hamartoma and axillary freckling	Common group	256 (86.1%)
The only treatment for leukemias in childhood is radiotherapy	Leukemia group	256 (86.1%)
Fever of unknown origin can be a symptom for childhood cancers	Leukemia group	254 (85.5%)

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Table III. *The incorrect answers most frequently given by the participants*

Questions	Group	Incorrect answers (N) (%)
The first examinations in a child with abdominal mass are hemogram and peripheral smear	Common group	64 (21.5%)
AFP level is significant in a child with a posterior mediastinum mass	Solid tumor group	66 (22.2%)
The definitive diagnosis of brain tumors in childhood is confirmed by cranial MR	Solid tumor group	90 (30.3%)
The diagnosis of neuroblastoma is established in children with abdominal mass, if VMA and HVA levels are high in urine	Solid tumor group	108 (36.3%)
Abdominal masses are the most common manifestation of malignant tumors in children	Solid tumor group	112 (37.7%)

AFP:Alpha-fetoprotein, VMA: Vanillylmandelic acid, HVA: Homovanillic acid

Mean age in the research group was 33.5±7.7(23-67). Leukaemia points, common question points and total points of the participants with respect to age group showed statistically significant differences (Mann-Whitney U test, p=0.033) (Table IV).

Table IV. *Comparison of leukemia, solid tumor, common group and total points according to age groups in the study*

Age groups		Leukemia group points	Solid tumor group points	Common group points	Total points
<33years old					
	Median	14 (77.7%)	30 (62.5%)	22 (64.7%)	66 (66%)
	Minimum	0 (0%)	4 (8%)	4 (11.7%)	8 (8%)
	Maximum	18 (100%)	48 (100%)	34 (100%)	100 (100%)
>34years old					
	Median	14 (77.7%)	28 (53.3%)	20 (58.8%)	62 (62%)
	Minimum	4 (22.2%)	0 (0%)	0 (0%)	6 (6%)
	Maximum	18 (100%)	48 (100%)	34 (100%)	100 (100%)
P*		**. 034**	. 244	**. 007**	**. 033**

*(*Mann-Whitney U test)*

The physicians younger than 33 years, which was the mean age, gave better answers to the leukaemia questions and the common questions (Mann-Whitney U test, p=0.034, p=0.007 respectively). Although, there was no significant difference between the points for solid tumour questions with respect to age of physicians (Mann-Whitney U test, p=0.244), there was significant difference between total scores (Mann-Whitney U test, p=0.033) (Table IV). Accordingly, the scores of young physicians were significantly higher. Those physicians who have been practising less than 10 years, were better especially with the common questions (Mann-Whitney U test, p=0.006) (Table V).

Table V. *Comparison of leukemia, solid tumor, common group and total points according to the duration of occupation of the participants*

Duration of occupation		Leukemia group points	Solid tumor group points	Common group points	Total points
<10years					
	Median	14 (77.7%)	28 (58.3%)	22 (64.7%)	64 (64%)
	Minimum	0 (0%)	4 (8%)	4 (11. 7%)	8 (8%)
	Maximum	18 (100%)	48 (100%)	34 (100%)	100 (100%)
>11years					
	Median	14 (77.7%)	28 (58.3%)	20 (58.8%)	60 (60%)
	Minimum	4 (22.2%)	0 (0%)	0 (0%)	6 (6%)
	Maximum	18 (100%)	48 (100%)	32 (94%)	98 (98%)
P*		. 131	. 265	**. 006**	**. 041**

*(*Mann-Whitney U test)*

There were statistically significant differences between the leukaemia, solid tumour, common group and total points with respect to the academic titles of the participants (Mann-Whitney U test, p=0.0001). Accordingly, the best answers in all groups were given by pediatrists and the lowest points were those of the general practitioners. Success limit was accepted as 75th percentile.

Accordingly, 88.8% of the pediatricians answered the leukaemia questions correctly. Their scores for solid tumour and common group questions were 66.6% and 64.7%, which were below the success limit. The scores of the physicians in all the other branches were below the success limit. When the academic titles of the participants were grouped as pediatricians and other physicians, the leukaemia, solid tumour, common group and total points showed statistically high significant differences (Mann-Whitney U test, p=0.0001) (Table VI).

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Table VI. *Comparison of the leukaemia, solid tumour, common group and total points when the academic titles of the participants are grouped as pediatricians and the others*

		Leukemia group points	Solid tumor group points	Common group points	Total points
Other physicians	**Median**	12 (66.6%)	24 (50%)	20 (58.8%)	58 (58%)
	Minimum	0 (0%)	0 (0%)	0 (0%)	6 (6%)
	Maximum	18 (100%)	48 (100%)	34 (100%)	100 (100%)
Pediatricians	**Median**	16 (88.8%)	30 (62.5%)	22 (64.7%)	68 (68%)
	Minimum	4 (22.2%)	10 (20.8%)	8 (23.5%)	24 (24%)
	Maximum	18 (100%)	48 (100%)	34 (100%)	100 (100%)
P*		**. 000**	**. 000**	**. 000**	**. 000**

The physicians whose specialist practice period was between 1-5 years were more successful in answering the leukaemia questions (Kruskal-Wallis test, p=0.007) and the common questions (Kruskal-Wallis test, p=0.003) than the physicians whose specialist practice period was >10 yeas. With respect to total points, there was no significant difference (Kruskal-Wallis test, p=0.052). The leukaemia, solid tumour, common group and total points of the participants with respect to having cancer diagnosis in the family and previous cancer follow-up showed statistically significant differences (Mann-Whitney U test, p=0.003).

4. DISCUSSION

The present study evaluated how much the symptoms of childhood cancers are recognised by the physicians in Istanbul, Turkey. As a result of the study, it was observed that the awareness of especially the general practitioners in Istanbul, Turkey on childhood cancers, primarily the solid tumours, was unsatisfactory. Although, mostly the pediatricians answered the questions correctly, they were not satisfactorily successful except for the leukaemia questions. The best acknowledged questions were the leukaemia questions while the least acknowledged ones were the solid tumour questions. The present study revealed that the knowledge and awareness of general practitioners on childhood cancers must be improved by implementing training programs as they are the first to see a patient.

No study has been conducted in Turkey yet, on how much the childhood cancer symptoms are known by physicians. The studies in the world literature on this subject are few and limited, being only on pediatricians or primary physicians [6-8]. The studies made worldwide and in Turkey on awareness concerning childhood cancer symptoms target cancer patients or the healthy population mostly [9,10]. The first study worldwide investigating the awareness of the physicians on childhood cancers was conducted in Brazil in 2007, targeting only general practitioners, with a small number of questions [11]. The mentioned study remained unsatisfactory, especially because the number of the questions asked was few. There were some other studies concerning pediatric oncology that evaluated the time between the start of the cancer symptoms and diagnosis [12,13]. One of the few such studies was conducted in Izmir, Turkey in 1997. In the mentioned study, contrary to what had been believed, the shortest diagnosis time was the brain tumours with 54 days, while the longest diagnosis time was Hodgkin's lymphoma with 199 days [13]. The results of the studies made on this subject worldwide are as follows: The time period between onset of symptoms and the confirming diagnosis was found to be on the average 21 days in neuroblastomas and 72 days in Ewing sarcoma conducted in the US in 1991 [14].

In most of the studies, the shortest diagnosis time is in the frequently observed tumours of the infancy, such as neuroblastoma, Wilm's tumour, while the longest diagnosis time is in brain tumours, bone and soft tissue tumours [15]. The two main factors that determine the time between the onset of the symptoms and diagnosis are the patient age and the type of the tumour. Pollock et al., demonstrated in their study that the time between the onset of the symptoms and diagnosis in younger children was shorter but longer in adultery [14]. The most extensive study is a digest published in Canada. According to this digest, the common point for all the studies is that the first reason for delay in the diagnosis of childhood cancers is the physician's delay. This is followed by causes such as delay of the patient and tumour type [16]. In a study conducted in Ghana in 2007, it was demonstrated that the cancers that lead to death most were lymphomas and brain tumours and that the diagnoses of the brain tumour patients who died were in the late stages [17]. This clearly shows the role of the physicians in early diagnosis of childhood cancers. The questions on solid tumours, for which the time between the onset of symptoms and diagnosis was the longest, were the questions which were least correctly answered in our study and the efforts to increase physician awareness concerning this issue are very few, both in Turkey and worldwide.

In a study in Brazil, it was seen that the awareness of the physicians is at a rather low level, not only for brain tumours but for all childhood cancers [11]. Pediatricians and physicians from other branches were included in the present study, while the study in Brazil included only general practitioners but not pediatricians. In the Brazilian study also the least correctly answered questions were the solid tumour questions.

In the current study, there was a significant difference between the awareness for cancer symptoms and sociodemographical characteristics such as the length of specialist practice and age. On the other hand, a significant difference could not be found between the awareness for cancer symptoms and both age and length of practice. No significant relationship could be found between the awareness for cancer symptoms and gender or marital status. The participant physicians who were younger than 33 answered more correctly the leukaemia and common

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questions. The best answered questions in the present study were the leukaemia questions. In leukaemias, 5-year survival is about 85%. This can be related to increased awareness of physicians on leukaemia and early diagnosis of leukaemia.

The knowledge inadequacy of the general practitioners, who were the primary care physicians for pediatric patients in Istanbul, can be clearly seen in the present study. In Turkey, the primary care training programs for childhood cancers, for which the 5-year survival rates are rather high if diagnosed early, are rather inadequate.

One of the main factors related to diagnosis delay is that there are no scan tests for childhood cancers [18]. Despite all these adversaries, early diagnosis in many childhood cancers can be given only by a well taken story and detailed physical examination [1,2]. In a study conducted in Manchester in 2009, more than half of the children with a story of

Li-fraumeni syndrome in the family was found to be positive for TP 53 mutation. The children displayed no syndromes and cancer developed in the follow-up of these children [19]. Similarly, there is the RB gene mutation in the diagnosis of retinoblastoma and there is mutation in the Ret protooncogene in multiple endocrine neoplasia type 1 and 2 (MENI and MEN II). In Turkey, all genetic tests cannot be made due to its high cost.

In the present study, leucocoria, which is one of the diagnostic findings of retinoblastoma, was answered correctly only by 70% of the participating physicians. This is a rather low rate for retinoblastoma, the 5-year survival rates are about 95% if diagnosed early. Early diagnosis for lymphoreticular malignities likely to develop may be possible when the existing immune deficiency of the patient at the time of application is assessed together with the family history [20]. Similarly, tendency to malignity increases in some genetic syndromes and syndromes that go together with immune deficiency (increase of the risk of leukaemia in Down syndrome patients, of lymphoreticular malignity and skin cancer risk in Bloom syndrome patients) [21].

It was observed that the physicians of all branches gave wrong answers to the questions on familial cancer syndromes and immune deficiency in our study. Although, the choice of the laboratory tests which will be requested concerning the symptoms at application to the physician is quite meaningful for cancer diagnosis, most of the time they do not provide decisive diagnosis, they can only assist the diagnosis as supporting finding. For a patient suspected of neuroblastoma, high level of neuron specific enolase (NSE) in blood and high level of vanillylmandelic acid (VMA), homovanillic acid (HVA) in urine will be meaningful but not decisive [22,23]. Similarly, although high beta human chorionic gonadotropin (HCG) is important in diagnosis and follow-up of germ-cell tumours, it is not a decisive diagnosis. High alpha fetoprotein (AFP) is also meaningful in the follow-up of germ-cell tumours and hepatocellular cancer [24,25]. In the present study, concerning this issue, the number of correct answers given to question 55, inquiring the relationship between neuroblastoma and levels of VMA and HVA, was 108 (36%). As can be seen, the awareness of both general practitioners and paediatricians is pretty low.

The first thing to do to increase physician's awareness in Turkey is to increase epidemiologic studies. As the briefing and training programs for the public and the physicians are rather inadequate in Turkey. In the present study, it was observed that physicians who had, in some way, confronted cancer, who had decided on a cancer diagnosis were more familiar with the symptoms of childhood cancers, without depending on branch, and answered the questions more correctly (Mann-Whitney U test, respectively p=0.0001).

In our study, physicians at or above the age 33 were less successful with leukaemia and common questions (Mann-Whitney U test, respectively p=0.034, p=0.007). In parallel with this, as the time of practice or the time of specialist practice for specialist physicians increased, the percentages of correct answers given to the questions decreased. That is, as age, time of practice and time of specialist practice increased, the number of wrong answers increased and levels of awareness decreased. Based upon these results, we can say that the trainings after becoming a specialist in Turkey are inadequate. There is no proficiency training program on this issue in Turkey yet. It may be possible to ensure early diagnosis in childhood cancers and provide renewal for physicians by distributing brochures-booklets containing the symptoms of childhood cancers for physicians to health institutions and establishments through the media and the television. Early consultation and information flow can be provided on suspected patients by making such efforts for especially general practitioners. Thus, childhood cancers that might be diagnosed early must not be neglected. Zitzelsberger et al., in a study conducted in 2004, asked family physicians questions on various adult cancers by telephone and e-mail; it was seen that the physicians built up awareness mostly by the internet and the things that could be done concerning this issue was discussed [26]. Another way to diagnose childhood cancers early is to build specific oncology centres for childhood cancers, such as leukaemia center, brain tumour center, solid tumour center. Although, the cost of such an implementation will be high, it will be a good step on the road to early diagnosis. This will ensure directing the patients especially applying to primary care physicians to the correct address. Some awareness trainings on childhood cancers are conducted for the public. The indirect effect of such trainings to increase physician awareness should not be neglected. Even if such efforts aim to raise public awareness in the first place, they will indirectly raise physician's awareness since the application of a conscious individual to the physician, his expectations from the physician and his questions to the physician will be high [27,28]. In a study conducted in Amsterdam in 2008, cancer symptoms were asked to the youth; it was demonstrated that the main symptoms, such as blood in the faeces, sudden development of a mass, sudden weight loss, were more effective in consulting a doctor but no importance was given to minor symptoms [29]. There is no study conducted in Turkey on this issue yet.

In the light of all these data, we can conclude that the trainings in Istanbul, Turkey on this issue, both before and after being a specialist are inadequate.

The present study included a limited number of physician population, representing the Marmara region, Turkey. So, more studies are needed to be done on the awareness of childhood cancers in different regions of Turkey with more participants. Turkey urgently needs training programs to raise the awareness of especially general practitioners working in primary care facilities on childhood cancers. Similar programs should be implemented for pediatricians too since they have remained under the average success level although they have been the group with the highest number of correct answers. Similarly, proficiency programs regarding childhood cancers must be developed for physicians who have not been able to catch up with new advancements as their practice times increase.

Compliance with Ethical Standards

Ethical Approval: The approval of the ethical board was given by the Ethical Board of the Medicine Faculty of the Marmara University(B.30.2.MAR.0.01.02/AEK/351)

Funding: The authors received no financial support for the study.

Conflict of interest: The authors declare that they have no conflict of interest.

Informed Consent: Informed consent was obtained from all participants.

Authors' Contributions: Concept and Design – MEC,GSB; Resources – MC; Materials – MEC,GSB; Data Collection and Processing – MEC; Analysis and Interpretation – MEC,GSB; Literature Search – MEC,GSB; Writing Manuscript – MEC;

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ANNEX 1

MARMARA UNIVERSITY, SCHOOL OF MEDICINE, DEPARTMENT OF CHILD HEALTH AND PEDIATRICS

SECTION 1: SOCIODEMOGRAPHIC CHARACTERISTICS

1. Gender : Female□ Male□

2. Age:

3. Marital Status: married □ single□

4. How long have you been practicing medical profession?

5. Academic title:

Pediatric Specialist □ General Practitioner □ Family Medicine Specialist □

Internal Medicine specialist□ Pediatric Surgery Specialist □

6. How many years have you been working as a specialist? 1-5 years □ 5-10 years□ >10 years□

7. Do you have any family member with cancer? Yes□ No□

8. Have you ever diagnosed with childhood cancer? Yes□ No□

9. If yes which type of cancer? Leukemia-lymphoma □ Solid tumor □ Brain tumor □

10. Have you ever followed-up a child with cancer? Yes□ No□

11 .If yes which type of cancer? Leukemia-lymphoma □ Solid tumor □ Brain tumor □

SECTION 2: QUESTIONS on CHILDHOOD CANCER

Questions	True answers (n)	(%)
12. Sudden developing weakness – fatigue can be a sign of cancer in a child	260	(87.5)
13. Sudden weight loss may be the first sign of cancer in a child	262	(88.2)
14. Axillary freckling and brown spots in the body do not require further examination if there is no other finding in a child	250	(84.1)
15. AFP level is significant in a child with a posterior mediastinum mass	66	(22.2)
16. Prognosis in girls is worse in childhood cancers	138	(46.4)
17. The definitive diagnosis of brain tumors in childhood is confirmed by cranial MR	90	(30.3)
18. Back pain can be the first sign of cancer in a child	222	(74.7)
19. In the physical examination, the palpable abdominal mass is the most common finding of cancer	129	(43.4)
20. In a child with night sweats, cancer must always be in the differential diagnosis	229	(77.1)
21. LDH elevation is one of the most significant markers in terms of cancer diagnosis	115	(38.7)
22. Leukopenia may be the first sign of cancer	245	(82.4)
23. The most common solid tumor of childhood is a brain tumor	148	(49.8)
24. Difficulty in urination may be a sign of cancer in a child	137	(46.1)
25. Childhood cancers are more common in girls	113	(38)
26. Depression may be a sign of cancer in a child	123	(41.4)
27. Abdominal masses are the most common manifestation of malignant tumors in children	112	(37.7)
28. AFP level should be considered in every child with anterior mediastinum mass	164	(55.2)

29. Cancer should be considered in a neonate with a sudden increase in head circumference	122	(41)
30. Evaluation of soft tissue masses in children is not urgent	238	(80.1)
31. The first differential diagnosis for lymphadenopathy not responding to antibiotic therapy is cancer	212	(71.3)
32. The most common childhood cancer is CNS tumors	176	(59.2)
33. Each child with cough lasting more than 2 weeks must have a chest X-ray for the diagnosis of a concomitant cancer	202	(68)
34. The prognosis of brain tumors in children is better than adults	181	(60.9)
35. Radiation therapy should be given to every childhood brain tumor	166	(55.8)
36. In a child with leukocytosis, the presence of nucleated erythrocytes in peripheral smear may be a sign of cancer	128	(43)
37. Fever of unknown origin can be a symptom for childhood cancer	254	(85.5)
38. No further investigation is needed in a child with iris hamartoma and axillary freckling	256	(86.1)
39. The risk of developing cancer in a child with a family history of malignancy at a young age is very high	226	(76)
40. Changes in sudden consciousness may be a sign of cancer in a child	228	(76.7)
41. The only treatment for leukemias in childhood is radiotherapy	256	(86.1)
42. Radiotherapy applied to brain tumors under the age of 9 causes falls in IQ	118	(39.7)
43. A child known to have retinoblastoma in the family should definitely be evaluated for cancer when presented with leg pain	235	(79.1)
44. Toxic granulation in a child with leukocytosis is a symptom of cancer	156	(52.5)
45. The first examinations in a child with abdominal mass are hemogram and peripheral smear	64	(21.5)
46. 2 weeks of vomiting accompanied with headaches is the definitive indicator of cancer in children	235	(79.1)
47 A child with constipation for >1 month should have pelvic imaging for cancer	149	(50.1)
48. Leucocoria is one of the most important signs of cancer in children	209	(70.3)
49. Unexplained unrest in the newborn may be a sign of cancer	112	(37.7)
50. Hemogram of leukocytes, erythrocytes, platelet series in the presence of one or more numerical-structural abnormality persists bone marrow aspiration biopsy should be done	252	(84.8)
51. Retinal pigment hypertrophy should be considered in the eye if there is a family history of familial adenomatosis poliposis	140	(47.1)
52. A child with a history of hearing loss and a family history of brain tumor should be evaluated in terms of familial cancer syndromes	240	(80.8)
53. Sudden hair loss may be a sign of cancer in the child	135	(45.4)
54. The most common manifestation of orbital tumors in children is proptosis	168	(56.5)
55. The diagnosis of neuroblastoma is established in children with abdominal mass, if VMA and HVA levels are high in urine	108	(36.3)
56. Unexplained bone pain is the definitive symptom of cancer in children	206	(69.3)
57. All series must be affected to diagnose cancer by looking at the hemogram	222	(74.7)
58. Stress is one of the most important etiologic causes of cancer in children	182	(61.2)
59. Children with hemihypertrophy must be followed with abdominal ultrasonography at least 18 years age	221	(74.4)
60. A child with normal hemogram and peripheral blood smear cannot have leukemia	202	(68)
61. Doctors should be careful in terms of brain tumours in children with headache and a history of skin cancer	192	(64.6)

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Obesity incidence is related to month of birth

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ABSTRACT

Objective: The relationship between the month of birth and obesity status of individuals has been claimed but not proven convincingly. We aim to provide more evidence towards the presumed relationship between month of birth and physical characteristics of individuals.

Materials and Methods: We used a Bio-impedance device to determine physical characteristics of 3,000 informed volunteers who attended our clinic.

Results: We found that the individuals who were born in the first three months of the year were heavier, taller and older than the applicants who were born in later months of the year.

Conclusion: We can conclude that the month of birth was significantly related to obesity incidence. We believe that this information will add to the knowledge on the importance of the relationship between birth month and physical and health characteristics of individuals living in a country with extreme seasonal temperatures.

Keywords: Body mass index, Weight, Fat content, Season

1. INTRODUCTION

Month of birth has been claimed to be correlated with the prevalence and incidence of many diseases and disorders [1, 2]. Even physical characteristics and psychological parameters claimed to be related to the month of birth [3, 4]. For example, a German group studied six million cardiovascular deaths between 1992 and 2007 and reported that deaths due to cardiovascular problems both in men and women were significantly less in individuals who were born in the month of May compared with the individuals who were born in other months of the year [5]. Benegas et al., investigated the effect of seasonal variation in mean systolic blood pressure in Spanish population and found that the greatest difference in systolic blood pressure occurred between adults born in spring (134.1 mmHg) and those born in autumn (140.3 mmHg) [6]. In another study by the same group, it was shown that male adults born in summer are 1.7 cm taller than their counterparts born in winter [3].

Around the globe, there are studies that used large data such as a Chinese study on 487,529 adults showing that the spring – and early summer-born adults have higher body mass index and waist circumference and shorter leg length than autumn – and winter-born adults [7]. Also, a UK study on 450,000 adults showed that season of birth is associated with birth weight, pubertal timing, adult body size and even educational attainment [4]. Similarly, a Japanese study used data from 69,693 children and showed that spring born children were taller and heavier than winter born children, but the prevalence of obesity did not vary with the season of birth [8]. A Polish study's finding on 1,241 children, though using a smaller sample than the Japanese study, found the opposite, i.e, children born in April to September were generally shorter and less heavy than those born in October to March [9]. A similar finding to the Polish study came from a South African study that investigated 1,165 adults and showed that individuals born in February to July were shorter and weighed less (by 13 to 17% of the standard deviation) than those born in August to January [10].

As briefly summarized above, many studies exist regarding the importance of the month of birth not only on the physical characteristics of individuals but also on the prevalence,

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incidence, and resistance to diseases and even on the success in schooling. However, the results of these studies do not exactly match. There may be confounding factors such as the hemispheric differences since when the Northern hemisphere is enjoying sunlight and heat in the months of May to September, the Southern hemisphere in the same time is going through winter. Despite this obvious difference in times of heat / sunlight, hemispheric differences for the cause of the above findings have been rejected by Henneberg and Louw on the grounds of finding similar effects of birth month on individuals living in the Southern and Northern hemispheres [11]. They have suggested that rather than the local heat / sunlight, a common factor for the entire globe, possibly related to the ellipsoid shape of the orbit of the planet, may be responsible [10].

Living in a country that enjoys extremes of temperature in seasons, we wished to examine the relationship between the months of births and obesity status of 3,000 individuals who attended our clinic.

2. MATERIALS and METHODS

This study was performed on outpatients who applied to our Sports Physiology clinic due to obesity from 2015 January to 2017 July. The study was approved by the Local Ethics Committee Three thousand subjects signed informed consent forms prepared by the human ethics committee. The heights of the subjects were measured while they were barefooted and back touching a wall. For each subject, we used a Bio-impedance device (Tanita BC418, Switzerland) to determine body weight, body mass index (BMI), fat percentage, fat weight and fat free mass (muscle weight). Mean and standard deviation of these values are shown in Table I. We have then formed a table to indicate the mean values of these variables against the month of birth (Table II).

Statistical Analyses

For each of the physical characteristics data we used Trendline analyses (4^{th} level Polynomial Trendline; Excel 2016). Trendline analysis is a linear least squares regression tool that can be employed to provide some correlation to data points that are seemingly not linked at all. Using this analysis tool, we built line of best fit graphs to illustrate concurrent effect of the month of birth on physical characteristics of individuals.

We have also used the z-test to see the dissipation between the average values of each month compared with the average value for the entire year for each of the variables. We used the following formula to obtain the z-value:

(Average value for one month – Average value for the year) / Standard Deviation value for the year

Significance level was set to $p<0.05$. We further examined the z-test values using a non-parametric test to illustrate the months of special importance for physical characteristics of individuals.

3. RESULTS

Examination of the 3,000 outpatients of our obesity clinic using Trend analysis we found significant relationships between the month of birth and age of the applicants; weight of the applicants; height of the applicants; BMI and fat free mass and fat mass values of the applicants. Age and month of birth, the height, the weight, fat weight, muscle weight, BMI, and fat percentage of the applicants are given in figures 1-7.

Table I. *Descriptive characteristics of individuals*

	Mean	SD
Age (year)	29.3	16.3
Height (cm)	157.5	22.6
Weight (kg)	89.7	109.2
BMI (kg/m^2)	35.2	21.6
%Fat	38.9	12.8
Fat weight (kg)	35	17.6
Muscle weight (kg)	51.8	17.7

Table II. *Z-test results for the entire data for 3,000 subjects. Z-value was obtained; for example for the distribution of height for the month of January as follows:*
Z = Average height value for January – Average height value for the entire year / Standard Deviation for height value for the entire year.

	Age	Height	Weight	BMI	Fat%	Fat Weight	Muscle Weight
January	0.18*	0.07**	0.1*	0.1*	0*	0.12*	0.07*
February	0.18*	0.15*	0.03	0.1*	0*	0	0.07*
March	0.12**	0.07**	0.1*	0.1*	0*	0.06**	0.07*
April	0.12**	0	0	0.1*	0*	0	-0.07
May	0.12**	0	– 0.03	0	0*	0	-0.07
June	– 0.06	0	– 0.03	0	0*	0	-0.07
July	– 0.24	0	– 0.06	0	-0.12	-0.06	-0.07
August	– 0.18	– 0.07	– 0.13	– 0.1	-0.12	-0.12	-0.13
September	0.06	0.07**	0.06**	0.1*	0*	0.06**	0**
October	-0.06	0.15*	0	0	-0.12	-0.06	0.07*
November	-0.12	0	-0.03	0	-0.12	-0.06	-0.07
December	-0.06	-0.07	-0.03	0	-0.12	-0.06	-0.07

In Table II, the highest z-values are indicated by the single asterisk and second highest z-values indicated by the double asterisks. As can be noted, first three months of the year had the highest z-values for all the variables tested. Furthermore, month of September also had high z-values.

A non-parametric Sign test was then used to distinguish significant birth months from the z-test table. For that we used the number of occurrences of the highest and second highest values for seven variables tested in each of the birth months. January and March had 7 out of 7 high z-values ($p<0.001$) and September had 6 out of 7 high z-values ($p<0.01$).

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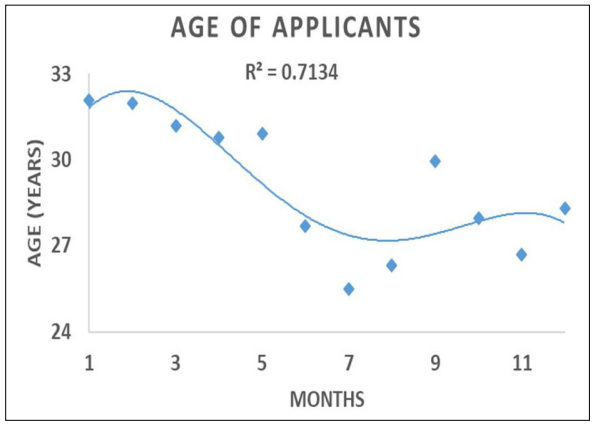

Figure 1. *Age of the applicants and month of birth were significantly correlated (R2 = 0.7134; p<0.01): As can be seen, the participants born in the first five months of the year were older when they applied to our obesity clinic compared with the persons born in the months from June to December.*

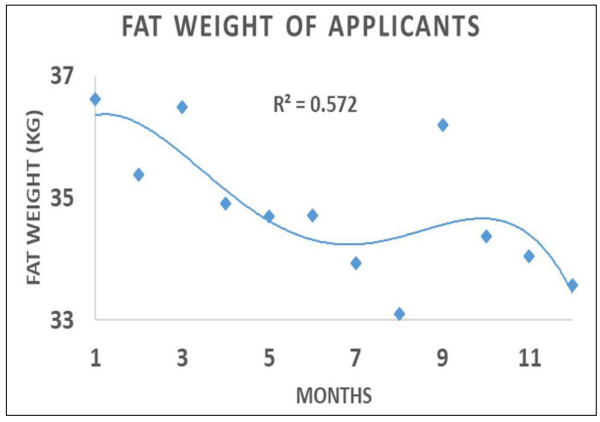

Figure 4. *The fat weight of the applicants also had one peak: Very similar to the finding in the weight of the applicants, fat weight also had one peak and that was occurring in the first three months of the year. The trend again was significant: R2 = 0.572 (p<0.05)*

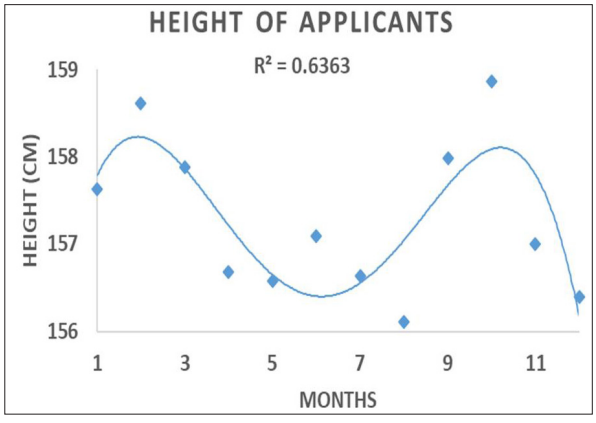

Figure 2. *The height of the applicants had two peaks: One peak in February and the other peak in October. The trend again was significant: R2 = 0.6363 (p<0.01)*

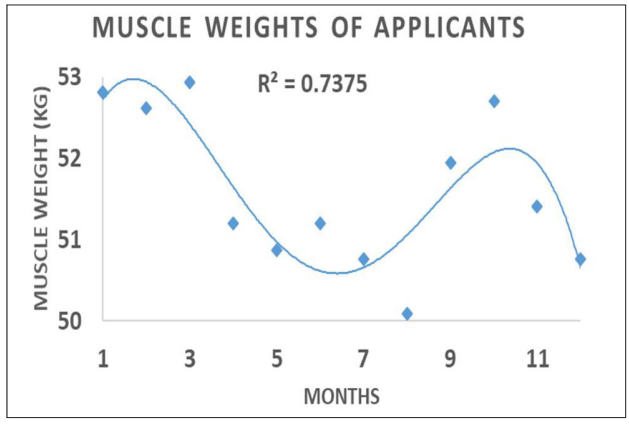

Figure 5. *Muscle weight of the applicants had two peaks: One peak in the first three months of the year and the other peak in October. The trend again was significant: R2 = 0.7375 (p<0.01). Similar to the Trendline curve for the height of the individuals.*

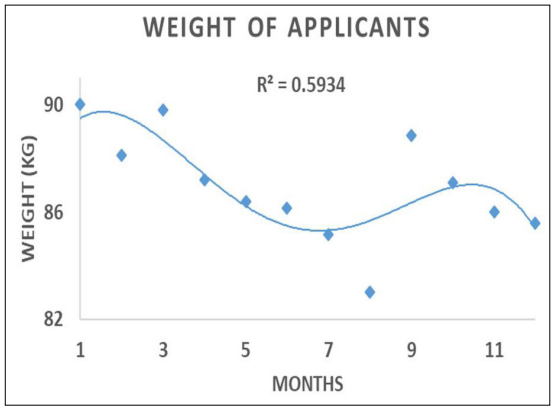

Figure 3. *The weight of the applicants had one peak: One peak in the first three months of the year. The trend again was significant: R2 = 0.5934 (p<0.05)*

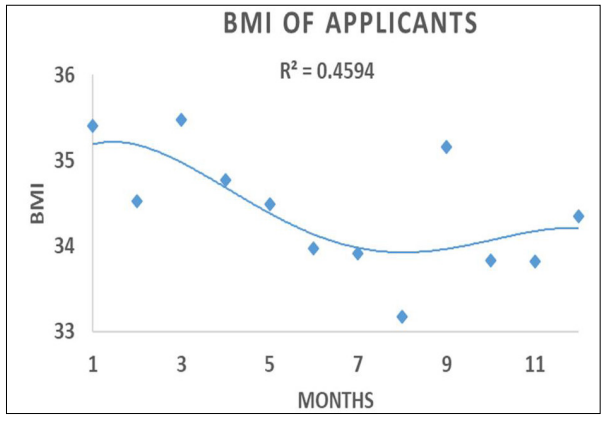

Figure 6. *BMI of the applicants had a single peak: There was only one peak in the first three months of the year. The trend again was significant: R2 = 0.4594 (p<0.05)*

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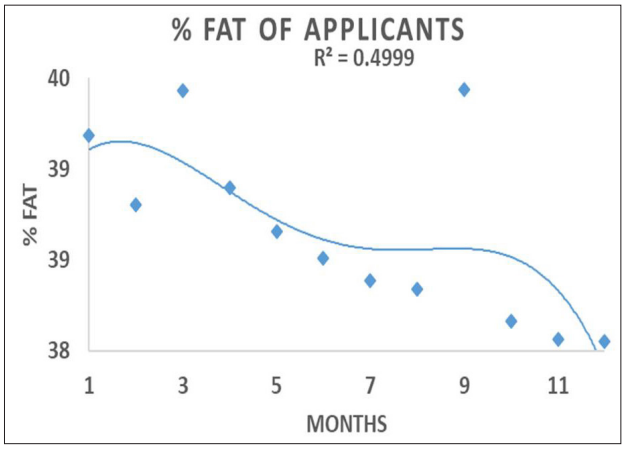

Figure 7. *Percentage fat of the applicants had one peak: The peak appeared in the first three month of the year. The trend again was significant: R2 = 0.4999 (p<0.05)*

4. DISCUSSION

We have two original findings to report in this investigation: Firstly, we found that the age of the applicants was the highest in the applicants who were born in early months of the year and again this number has reduced in applicants who were born in later months of the year. Secondly, the subjects who were born in the early months of the year were taller, heavier and had more muscle and fat mass than the subjects born in other months of the year.

Older age applicants in early months of the year: This finding is difficult to explain since the age of applicants is not expected to vary with months of the year. However, the fact that birth years are utilized to calculate the age of individuals may introduce a slight error in the age calculations since at any time of the year the January born individuals should be "older" than others who are born in later months of the year. This calculation error strengthens our findings further as we have found that January born individual were older when they have applied to our clinic. The only explanation that we can think of for this unusual finding is the suggestion that self-awareness increases with age [12]. It has been reported by a group of researchers in Tanzania that obesity awareness increases with the age of individuals and older people become more self-aware than the younger individuals regarding body fat [12]. Therefore, it is not surprising that the older individuals are more aware about the consequence of being fat and hence may respond to our clinic to find a solution to their weight problem.

Taller, heavier, and more obese applicants are born in the early months of the year: Comparison of our findings with that of the previously reported height and obesity incidences vary very much. For example, a Chinese study showed that spring – and early summer-born adults had shorter legs, higher body mass index and wider waist circumference [7]. A Japanese study showed that spring born children were heavier and taller than winter born children, but the prevalence of obesity did not vary with the season of birth [8]. A Polish study found the opposite, i.e., children born in April to September were generally less heavy and shorter than those born in October to March [9]. A somewhat similar finding to the Polish study came from a South African study that showed that individuals born in February to July were weighed less (by 17%) and shorter than those born in August to January [10]. A British study showed that in men, BMI and the prevalence of obesity (BMI > or = 30 kg/m2) varied as a function of birth month and was greater among those born between January to June period than among those born in between July to December [13]. Benegas et al. have shown that male adults born in summer are 1.7 cm taller than their counterparts born in winter [3].

Our findings are similar but not the same as the Polish, British and South African studies where early month of the year was included in the birth month for more obese, heavier and taller adults. Therefore, there must be other factors underlying the current finding than simply to the seasonal temperature variation since during January, Turkey is in the middle of winter while South Africa is enjoying the height of summer. It is well-known that the environmental conditions (food, temperature and rainfall) in the Southern Hemisphere six months out of phase from those in the Northern Hemisphere. Consequently, the similarity of our findings to those from the Southern Hemisphere suggests a factor common for the entire globe, possibly related to the ellipsoid shape of the orbit of our planet around the sun [11]. It is well-known that the earth has an ellipsoid orbit around the sun and the sun is positioned in one of the two focal points of earth's ellipsoid orbit. The world comes closer to the sun in the Northern winter / Southern summer months and hence it is axiomatic that it would receive more energy and babies born in these months may be fed from food that are richer in nutrients compared with the babies born in later months of the year [10, 11]. Furthermore, it has been shown that the infant weight gain in the first week of life is related to overweight at age 2 [14]. Therefore, overweight babies born in the high energy months may be heavier when they reach adult ages [15].

Conclusion

We found that the month of birth was significantly related to obesity incidence. Individuals born in early months of the year were heavier, taller and more obese than individuals born in later months of the year. This information adds to the knowledge on the importance of the relationship between birth month and physical and health characteristics of individuals living in a country with extreme seasonal temperatures. This information can be useful in predicting the health and welfare status of individuals and their awareness on their conditions.

Limitations of study: The study examines only the obese people who applied to our clinic. It does not include non-obese individuals. Lack of simple statistical analysis may be the limiting factor of the study.

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Compliance with Ethical Standards

Ethical Approval: The study protocol was approved by the Local Ethics Committee.

Financial Support: The author has no relevant financial information to disclose.

Conflict of Interest: The author has no potential conflicts to disclose.

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Antioxidant and antimicrobial activity of *Ficus sycomorus* fruit and leaf extracts

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ABSTRACT

Objective: The aim of this study was to determine the antioxidant and antimicrobial activity of *Ficus sycomorus* fruit and leaf extracts obtained from the Turkish Republic of Northern Cyprus.

Materials and Methods: Fruits and leaves of *F. sycomorus* were collected from the Kyrenia region of Northern Cyprus in July 2018. The leaf and fruit samples were extracted with the distilled water, methanol, ethanol, acetone and chloroform solvents (1:10 [w/v]). After evaporation, samples were suspended in methanol at the final concentration of 100 mg/mL. The antimicrobial activity of the leaf and fruit extracts was evaluated using the Kirby-Bauer Disk Diffusion Method. Total phenolic content (TPC) and total flavonoid content (TFC) of the extracts were determined using the methods reported by Stankovic in 2011 and Sharm and Vig in 2013. Antioxidant activity of samples was tested using free radical scavenger method.

Results: The leaf extracts of *F. sycomorus* was active. The inhibition zone diameters ranged from 1.8mm to 13.00 mm. Fruit extracts and methanol controls showed no inhibitory effect on strains. However, bacteriostatic activity against *Enterococcus faecalis* was observed in fruit-water extract. The highest antimicrobial activity was shown against *Staphylococcus aureus* (13 mm) for ethanolic extracts at 100 mg/mL concentration. Minimal inhibition concentration (MIC) for ethanolic extract was observed starting at 25 mg/mL concentration against *S. aureus*. Although, no antimicrobial activiy was observed in fruit extracts, the highest 2,2-diphenyl 1-picrylhydrazyl (DPPH) activiy and phytochemical content were recorded in fruit extracts.

Conclusion: These results demonstrate that leaf extracts of *F. sycomorus* can be used as a curative agent for the treatment of *S. aureus* and fungal infections and may be effective against pathogenic microorganisms that are resistant to antibiotics. Antioxidant content of fruit and leaf extracts can be effective against the negative effects of free radicals.

Keywords: Antioxidant activity, *F. sycomorus, S. aureus*

1. INTRODUCTION

Ficus sycomorus belongs to the Moraceae, which is a family of flowering plants, containing about forty genera and more than thousand species. This family is the best commonly found in tropical and subtropical areas and is often referred to as the mulberry family or the fig family [1]. The plant is indigenous to African countries and mostly grows well in tropical countries like Oman. It also grows in the Arabian Peninsula and in Lebanon. It is also found in Cyprus, Madagascar, Israel and Egypt. The plant grows to a height of about 10 to 20 m. The branches of the plant begin from the lower part of the body and form shapes like umbrellas. The *F. sycomorus* leaves are dark green, yellow-veined, heart-shaped and about 10 to 14 cm long. The diameter of the fruits is about 2 to 3 cm and round. The fruits are green when they are raw, and they become yellow or red when they ripen [2]. In Northern Cyprus, the *F. sycomorus* fruit is known as 'Cümbez' 'Pharaoh fruit' among the people. The tree of cümbez is known to give fruit seven times a year. When the tree gives fruit, the fruit is scratched with a knife and the fruit is mature. Scratched fruits ripen after about 7-10 days and become ready to be consumed. The most well-known *F. sycomorus* plant in Northern Cyprus is located in the courtyard of the Lala Mustafa Paşa Mosque in Famagusta. The mosque was originally built as the Cathedral of Saint Nicholas. It is estimated that the tree was erected in 1298 when the construction of the cathedral began.

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The height of the tree is 15 meters and the estimated age is 715. The body of the tree is surrounded by smaller branches growing from the main body. The body is divided into 7 branches after 2.70 meters. Each branch around the main body is said to have coincided with a century. It is the oldest and most vivid tree in Cyprus. The fact that this tree is the oldest tree in the history of the all island and witnessed many events from the past to the present make the historical Cümbez tree in this region culturally important [3, 4].

Extracts of fruit, leaf, root and stem bark of the plant are used to treat various ailments such as cough, diarrhea, skin infections, jaundice, snake bites, chest diseases, cold, dysentery, stomach disorders, lactation disorders, liver disease, epilepsy, tuberculosis, helminthiasis, infertility, and diabetes mellitus [5, 6].

Constantly developing technology, environmental pollution, contaminated waters, radiation, heavy metals, pesticides and oxygen metabolism in living cells cause the formation of free radicals in the human body [7]. Free radicals are known to cause many diseases, particularly cancer. Antioxidants protect our body against all damages caused by free radicals that threaten human health. The importance of foods containing antioxidants should be known and consumed in order to prevent the spread of cancer disease in Cyprus and all over the World. Another important problem is the resistance of bacteria to antibiotics. Nowadays, all around the world, exploratory work is going on to find effective solution against drug resistant bacteria [8]. The discovery of new antimicrobials through plants provides new approaches and benefits for minimizing antibiotic resistance. In many studies, it has been mentioned that *Ficus* species have potential antibacterial activity [9].

The facts that this important Cypriot plant has a value to the culture it belongs to, and that it is rare and not widely known and that there is no study that has been conducted on it in North Cyprus or in Turkey make this study worthwhile and valuable.

This study aimed to screen phytochemical properties and pharmacological activities of leaf and fruit extracts of *F. sycomorus*. The leaf and fruit extracts of the plant were prepared for the analysis using acetone, ethanol, pure water and methanol solvents. The minimum inhibitory concentration (MIC) regarding the antimicrobial activity of these medicinal plants was also examined.

2. MATERIALS and METHODS

Chemicals, Culture Media and Antibiotics

Ethanol, methanol, chloroform, acetone, 2,2-diphenyl-1-picrylhydrazyl (DPPH), sodium nitrite ($NaNO_2$) and sodium hydroxide (NaOH) were purchased from Sigma-Aldrich, (Germany). Mueller Hinton Agar Butylated hydroxytoluene (BHT), Folin-Ciocalteu reagent, sodium bicarbonate ($NaHCO_3$) and gallic acid standard were purchased from Merck KgaA, (Germany). Aluminum chloride hexahydrate ($AlCl_3H_{12}O_6$), Phoenix ID Broth and Nystatin (Oxoid, 100 units) were purchased from ACROS, BD Phoenix and Oxoid (United Kingdom). Blank antimicrobial discs, tetracycline (Bioanalyse Limited, 30 µg), ciprofloxacin (Bioanalyse Limited, 5 µg) and teicoplanin were purchased from (Bioanalyse Limited, 30 µg) Bioanalyse Limited, (Turkey).

Collection and Preparation of Plant Material

Fruits and leaves of *F. sycomorus* were collected from the Kyrenia region of Northern Cyprus in July 2018. The collected fruit and leaves were washed by distilled water to remove dust and soil and then dried. The washed fruits were cut into thin slices with a knife and dried in a food dehydrator machine and the washed leaves were dried at room temperature. The dried leaves and fruits were grounded with an electric mixer and stored in a +4°C refrigerator until the day of use in the laboratory.

Preparation and Extraction of Leaf and Fruit Extracts

The leaf and fruit samples were extracted with the distilled water, methanol, ethanol, acetone and chloroform solvents (1:10 [w/v]) in the shaker for 72 hours at room temperature. Solvents, after being filtered by Wattman No. 4 paper (Camlab, UK), were evaporated and then samples were suspended in methanol at the 100 mg mL^{-1} final concentration. The extracts were kept refrigerated at +4°C for phytochemical analysis for the antioxidant, and antimicrobial activity.

Antimicrobial Test

The antimicrobial activity of the leaf and fruit extracts were evaluated by the Kirby-Bauer Disk Diffusion Method [10]. A total of 1 fungal and 7 bacteria species (*Escherichia coli*, *Enterobacter cloacae*, *Klebsiella* spp., *Bacillus subtilis*, *Enterococcus faecalis*, *Staphylococcus aureus*, *Staphylococcus epidermidis* and *Candida albicans*) were used for the antimicrobial test. The antibacterial activity was performed on Mueller Hinton Agar. 10 µL of the microbial suspension was taken with a pipette and transferred to the center of Mueller Hinton agar and then spreaded homogeneously on the surface with a wooden cotton applicator stick. The sterile antimicrobial blank discs impregnated with 20 µl of the extracts were strategically placed away from each other. Methanol solvent and standard antibiotics were used as negative and positive controls, respectively. Tetracycline for *B. subtilis, S. aureus* and *S. epidermidis;* Ciprofloxacin for *E. coli, E. cloacae* and *Klebsiella* spp.*;* Nystatin for *C. albicans* and Teicoplanin for *E. faecalis* were used as the positive controls. The plates were kept at room temperature for 20-30 minutes. Then, the plates were placed in the incubator for 18-24 hours at 35°C. Following the incubation, the inhibition zones around the discs were evaluated. MIC of the extracts that showed antibacterial activity against test microorganisms was determined. This analysis was performed based on fact that the lowest inhibitory concentration determines the effectiveness on the test microorganisms (*S. aureus* and *C. albicans*). In this test, the 12.5, 25, 50, 75 and 100 mg mL^{-1} concentrations of extracts were investigated for their inhibitory effects against various microorganisms.

Total Antioxidant Test

The antioxidant activity of extracts were determined by DPPH Radical Scavenging Method. This method is based on the reduction of DPPH, a dark violet color compound and the absorbance reduction was measured by Ultraviolet-Visible (UV-Vis) spectrophotometer [11]. The antioxidant activities of the extracts, which were expressed as the activity of capturing free radicals, were determined by the use of DPPH radicals according to the method of Yılmaz, and Türkmen, et al. [12, 13]. DPPH radicals (0.025 g/L) prepared in 3.9 mL methanol was added to 100 µL of the extracts. The mixture was incubated at room temperature and in the dark for 30 minutes. In this analysis based on the opening of purple color of the DPPH solution, the residual amount of DPPH was measured at 515 nm by using spectrophotometer. Inhibition of DPPH was calculated as percent by the following formula. All analyzes were repeated 3 times. For the Control value: Methanol + DPPH, For Blank: Methanol, Against Blank (methanol): Methanol + DPPH (control), Against Blank: Plant sample + DPPH were used. BHT at 200 µg mL^{-1} concentration was used as standard antioxidant substance.

Inhibition % = [(Control Absorbance – Sample Absorbance / Control Absorbance)] × 100

Total Flavonoid Content (TFC)

According to the method reported by Sharma and Vig (2013), 1 mL of extracts were diluted with 5 mL of distilled water [14]. 0.3 mL $NaNO_2$ (5%) was added to the samples and incubated for 5 min at room temperature. Then, 0.6 mL of $AlCl_3.6H_2O$ (10%) was added to the mixture and after incubation under the same conditions, 2 mL of 1M NaOH was added and the final volume of reaction mixture was completed to 10 mL with distilled water. The absorbance of the prepared mixtures was determined spectrophotometrically at 510 nm. Routine equivalent standard was used by solving in distilled water at 0-125 mg/mL concentrations. All analyzes were repeated 3 times and calculated according to the slope value (y = 10,954x). TFC was expressed as mg routine equivalents (mg RE/g) per gram.

Total Phenolic Content (TPC)

Soluble phenolic content of fruit and leaf extracts were determined using Folin-Ciocalteu reagent. 0.5 mL of extracts were incubated in a water bath at 45°C for 45 minutes with the addition of 2.5 mL of Folin-Ciocalteu reagent (10%) and 2.5 mL of $NaHCO_3$ (7.5%). The absorbance of the mixtures was measured spectrophotometrically at 765 nm. According to the calibration graph using gallic acid as standard, the TPC is expressed as mg gallic acid equivalents (mg GAE/g) per gram [15]. Gallic acid standard was used by solving it in distilled water at 0-150 µg/mL concentrations. All analyzes were repeated 3 times and calculated according to the slope value (y = 8,8286x).

Statistical Analysis

Statistical analysis was performed for antioxidant activity, TFC and TPC results. In order to determine significant differences between the samples, the software SPSS 22.0 for Windows (SPSS Inc., Chicago, IL, USA) was used. Variance analysis (ANOVA) and Tukey multiple comparison tests were performed. Each spectrophotometric analysis was repeated at least three times. ($P<0.05$).

3. RESULTS

The range of the percentage extraction yield of the leaf and fruit extracts was very extensive: from 4.8% to 51.65% (Table I). The highest yield was calculated as 51.65% and 32.45% in the methanolic and aqueous fruit extracts. However, the yield of the leaf extracts were lower than that of fruit samples. This value for leaf extracts ranged from 4.8% to 11.4%. For both fruit and leaf samples, the high yield was observed in methanolic extracts.

Negative control (methanol) showed no inhibitory effect on strains. All leaf extracts displayed no antibacterial activity against *B. subtilis*, *S. epidermis*, *E. coli, Klebsiella* spp., *E. cloacae* and *E. faecalis*. However, the prepared fruit samples showed no inhibitory effect on tested microorganisms, except on *E. faecalis*. Aqueous fruit extract had bacteriostatic activity on this bacteria (Table IV), (Figure 3). As seen from Table II, leaf aceton, methanol and ethanol extracts had only antibacterial activity against *S. aureus*. The most effective extract on *S. aureus* was ethanolic leaf sample with the 13 mm zone diameter (Table II; Figure 1). This activity was lower than inhibition diameter of tetracycline positive control (23 mm). Remarkable antifungal activity of acetone and ethanol leaf extracts was observed on *C. albicans* (Table III; Figure 2). While control of nystatin resulted in 15 mm against *C. albicans*, the effect of extracts ranged between 10mm and 12 mm. MIC analysis was performed for leaf acetone, methanol and ethanol extracts showing antimicrobial activity on *S. aureus* and *C. albicans*.

Table I. *Percentage yield of leaf and fruit extracts*

Name of Extract	Amount of leaf and fruit extracts after evaporation (g)	Percent yield of leaf and fruit extracts (%)
Leaf-Water	0.85	8.5
Leaf-Methanol	1.14	11.4
Leaf-Ethanol	0.53	5.3
Leaf-Chloroform	0.53	5.3
Leaf-Acetone	0.48	4.8
Fruit-Water	6.49	32.45
Fruit-Methanol	10.33	51.65
Fruit-Ethanol	5.48	27.4
Fruit-Chloroform	0.68	3.4
Fruit-Acetone	0.97	4.85

Table II. *Diameter of the inhibition zone (mm) of the leaf extracts (100 mg/mL concentration, 20 µL) against B. subtilis, S. aureus and S. epidermidis*

Microorganisms tested	Leaf-Acetone	Leaf-Chloroform	Leaf-Methanol	Leaf-Ethanol	Leaf-Water	Methanol (NC)	Tetracycline (PC)
B. subtilis	-	-	-	-	-	-	25
S. aureus	11	-	10	13	-	-	23
S. epidermidis	-	-	-	-	-	-	14

(-) represents a no inhibition zone against microorganism. PC: Positive control, NC: Negative control

Table III. *Diameter of the inhibition zone (mm) of the leaf extracts (100 mg/mL concentration, 20 µL) against C. albicans*

Microorganisms tested	Leaf-Acetone	Leaf – Chloroform	Leaf-Methanol	Leaf-Ethanol	Leaf-Water	Methanol (NC)	Nystatin (PC)
C. albicans	10	-	-	12	-	-	15

(-) represents a no inhibition zone against microorganism. PC: Positive control, NC: Negative control

Table IV. *Diameter of the inhibition zone (mm) of the fruit extracts (100 mg/mL concentration, 20 µL) towards E. faecalis*

Microorganisms tested	Fruit-Acetone	Fruit – Chloroform	Fruit-Methanol	Fruit-Ethanol	Fruit-Water	Methanol (NC)	Teicoplanin (PC)
E. faecalis	-	-	-	-	1.8	-	19

(-) represents a no inhibition zone against microorganism. PC: Positive control, NC: Negative control

Table V. *Minimum inhibitory concentration (MIC) values of leaf-acetone, leaf-ethanol and leaf methanol extracts against the S. aureus*

	Leaf-Acetone (12.5 mg/mL)	Leaf-Acetone (25 mg/mL)	Leaf-Acetone (50 mg/mL)	Leaf-Acetone (75 mg/mL)	Leaf-Acetone (100 mg/mL)	Methanol (NC)	Tetracycline (PC)
S. aureus	-	-	-	9	11	-	24
	Leaf-Ethanol (12.5 mg/mL)	**Leaf-Ethanol (25 mg/mL)**	**Leaf-Ethanol (50 mg/mL)**	**Leaf-Ethanol (75 mg/mL)**	**Leaf-Ethanol (100 mg/mL)**	**Methanol (NC)**	**Tetracycline (PC)**
	-	9	11	13	13	-	26
	Leaf-Methanol (12.5 mg/mL)	**Leaf-Methanol (25 mg/mL)**	**Leaf-Methanol (50 mg/mL)**	**Leaf-Methanol (75 mg/mL)**	**Leaf-Methanol (100 mg/mL)**	**Methanol (NC)**	**Tetracycline (PC)**
	-	-	-	8	10	-	24

(-) represents a no inhibition zone against microorganism. PC: Positive control, NC: Negative control

Table VI. *Minimum inhibitory concentration (MIC) values of leaf-acetone and leaf ethanol extracts against the C. albicans*

	Leaf-Acetone (12.5 mg/mL)	Leaf-Acetone (25 mg/mL)	Leaf-Acetone (50 mg/mL)	Leaf-Acetone (75 mg/mL)	Leaf-Acetone (100 mg/mL)	Methanol (NC)	Nystatin (PC)
C. albicans	-	-	-	9	10	-	13
	Leaf-Ethanol (12.5 mg/mL)	**Leaf-Ethanol (25 mg/mL)**	**Leaf-Ethanol (50 mg/mL)**	**Leaf-Ethanol (75 mg/mL)**	**Leaf-Ethanol (100 mg/mL)**	**Methanol (NC)**	**Nystatin (PC)**
	-	-	10	11	12	-	13

(-) represents a no inhibition zone against microorganism. PC: Positive control, NC: Negative control

Table VII. *Total phenolic content (TPC), Total flavonoid content (TFC) and antioxidant activity (DPPH scavenging) of different leaf extracts of Ficus sycomorus*

Sample	TPC (mg GAE/g)	TFC (mg RE/g)	DPPH (%)
Leaf-water	*3.72±0.08^{b}	*0.19±0.015^{c}	*1±2.55^{d}
Leaf-acetone	*2.55±0.38^{c}	*1.38±0.306^{a}	*33±3.38^{b}
Leaf-chloroform	*7.09±0.23^{a}	*1.24±0.064^{ab}	*42±0.13^{a}
Leaf –ethanol	*2.23±0.00^{c}	*1.37±0.246^{a}	*18±0.13^{c}
Leaf –methanol	*2.38±0.09^{c}	*0.84±0.107^{b}	*47±2.17^{a}
BHT (200 µg mL^{-1})			*26.29±0.18^{e}

*Values are mean ± Standard Deviation (SD) of three replicate analyses. 100 mg/mL concentration was used for the tests. *(The presented datas are mean of triplicate determinations (n=3), ± Standard Deviation. The difference between the values expressed by the different symbols in table (a-c, and a-e) is significant (P<0.05)). BHT: Butylated hydroxytoluene*

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Table VIII. *Total phenolic content (TPC), Total flavonoid content (TFC) and antioxidant activity (DPPH scavenging) of the different fruit extracts of Ficus sycomorus*

Sample	TPC (mg GAE/g)	TFC (mg RE/g)	DPPH (%)
Fruit-water	*5.62±0.05[c]	*0.07±0.004[d]	*76±2.23[b]
Fruit-acetone	*11.29±0.39[a]	*1.38±0.021[a]	*86±0.06[a]
Fruit-chloroform	*7.78±0.33[b]	*1.08±0.058[b]	*69±1.21[c]
Fruit-ethanol	*1.93±0.27[d]	*0.32±0.009[c]	*86±4.78[a]
Fruit –methanol	*1.91±0.33[d]	*0.12±0.013[d]	*86±0.19[a]

*Values are mean ± Standard Deviation (SD) of three replicate analysis. 100 mg/mL concentration was used for the tests. *(The presented datas are mean of triplicate determinations (n=3), ± Standard Deviation. The difference between the values expressed by the different symbols in table (a-d and a-c) is significant (P<0.05)).*

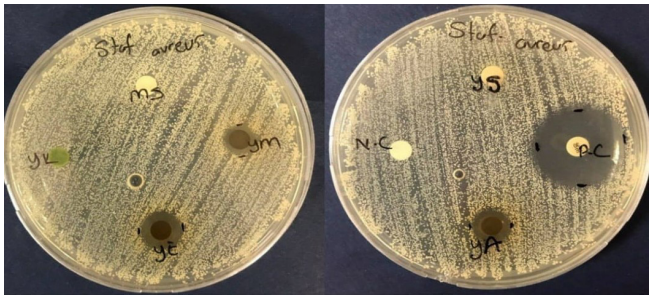

Figure 1. *Inhibition zone of leaf-acetone (YA), leaf-methanol (YM) and leaf-ethanol (YE) extracts against S. aureus*

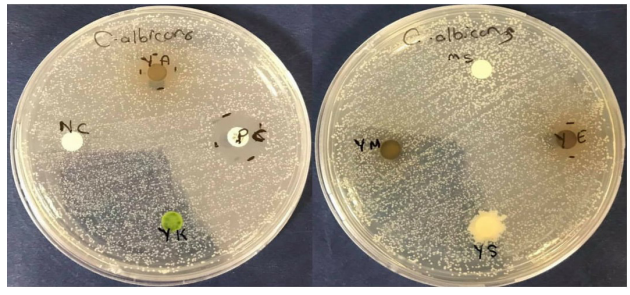

Figure 2. *Inhibition zone of leaf-acetone (YA) and leaf-ethanol (YE) extracts towards C. albicans*

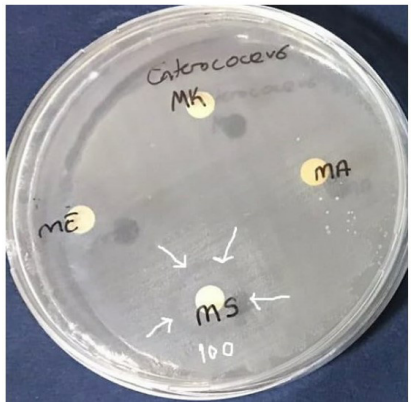

Figure 3. *Bacteriostatic activity of fruit-pure water (MS) extract towards E. faecalis, no inhibition zone of other fruit and leaf extracts*

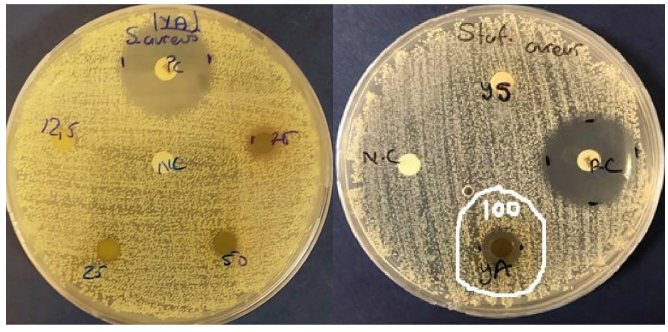

Figure 4. *The inhibition zone towards S. aureus at different concentration of the leaf-acetone extracts (YA)*

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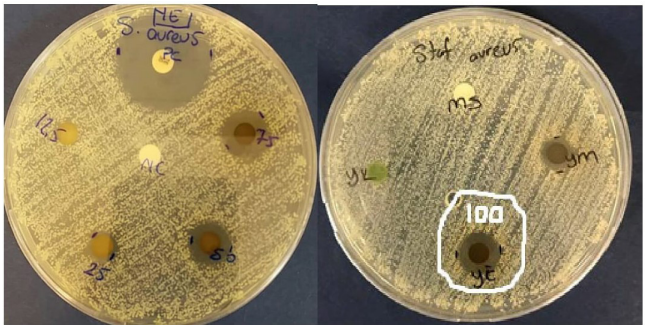

Figure 5. *The inhibition zone towards S. aureus at different concentration of the leaf-ethanol extracts (YE)*

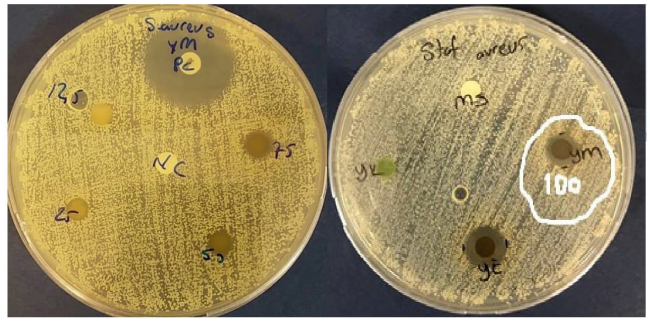

Figure 6. *The inhibition zone towards S. aureus at different concentration of the leaf-methanol extracts (YM)*

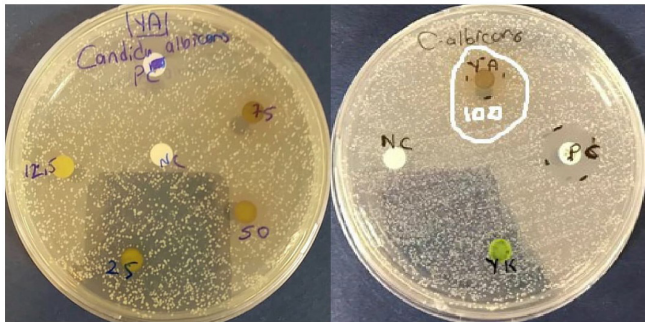

Figure 7. *The inhibition zone towards C. albicans at different concentration of the leaf-acetone extracts (YA)*

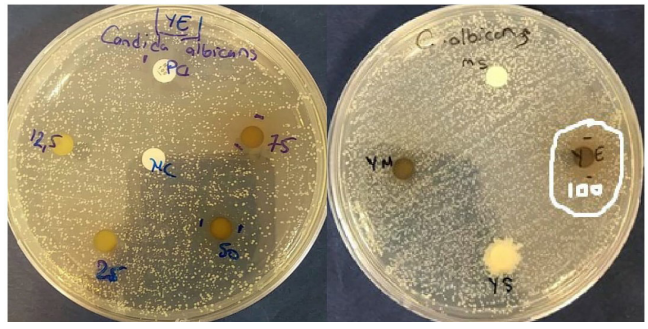

Figure 8. *The inhibition zone towards C. albicans at different concentration of the leaf-ethanol extracts (YE)*

Minimum inhibitory concentration value for leaf acetone was observed starting at 75 mg/mL concentration against *S. aureus* and *C. albicans* (Table V-VI; Figure 4, 7). MIC values of ethanolic and methanolic leaf extracts were 25 and 75 mg/mL on *S. aureus* (Table V; Figure 5, 6). MIC value (50 mg/mL) of ethanolic leaf extract observed against *C. albicans* was lower than that of acetone sample (Table VI; Figure 8).

In this present study, all quantitative examination of phytochemical analysis and DPPH were found statistically significant ($P<0.05$). As can be seen in Table VII-VIII, TPC and TFC of the extracts ranged from 1.91±0.33 to 11.29±0.39 mg GAE/g; from 0.07±0.004 to 1.38±0.306 mg RE/g, respectively. High TPC value was calculated as 11.29±0.39 mg GAE/g in the acetone fruit extract. However, the lower TPC values ranging from 1.93±0.27 to 1.91±0.33 mg GAE/g were obtained from ethanolic and methanolic extracts. Highest TFC was identified in acetone leaf and fruit extracts, 1.38±0.306 and 1.38±0.021 mg RE/g. The TFC was noted as minimum in aqueous fruit extract (0.07±0.004). In leaf extracts, chloroform extract was the highest TPC value (7.09±0.23). Chloroform has the least polarity index in the test solvent used but which yielded the highest phenolic substances. This may be due to the high hydrophobicity of the compounds. The results of flavonoids for all leaf samples were lower than the results of phenolic substances. This is because flavonoids are the subgroup of phenolics.

2,2-diphenyl-1-picrylhydrazyl radical scavenging activity was evaluated by comparing it with the standard antioxidant activity of BHT. The DPPH activity of *F. sycomorus* samples was not comparable with that of BHT testing at 200 µg mL^{-1} concentration. The antioxidant activity of DPPH was observed in the leaf extracts ranging from 1±2.55% to 47±2.17%. TPC and TFC values of leaf methanolic extract showed the highest antioxidant activity (47±2.17%) but still they were lower than the values of chloroform and acetone extracts that had the maximum contents. Even though the low DPPH activity (1±2.55%), aqueous leaf extract had the high TPC content (3.72±0.08). The free radical scavenging capacity of fruit extracts was higher than that of the leaf extracts. Based on the polarity of solvents, the highest antioxidant activity of DPPH for fruit samples was recorded as 86% in methanol, ethanol and acetone extracts. According to Table VII-VIII, inhibition % values ranged from 1±2.55% to 86±0.06. According to these results, the presence of antioxidant compounds of fruit and leaf extracts could be effective against the negative effects of free radicals. Our results also indicated the effect of extraction solvents on antioxidant activity and phytochemical contents in bioactivity studies of plants.

4. DISCUSSION

Four Gram-positive, three Gram-negative bacterial strains and one fungal strain were tested for antimicrobial activity of the extracts. Extracts did not show any inhibitory activity against three Gram-negative bacterial strains. However, a moderate activity was observed against one of the four Gram-positive bacterial strains, *S. aureus* and one fungal strain (Table II-III). The inhibition zone to *S. aureus* and *C. albicans* at concentrations of 25, 50, 75, 100 mg/mL and 50, 75, 100 mg/mL of leaf extract was in the range of 8-13 mm and 9-12 mm, respectively (Table V-VI). The antimicrobial activity of fruits and leaves of *F. sycomorus* extracts differentiated according to the species of microorganisms tested, the solvents used and extraction process.

Ghareeb et al., reported leaf-methanol extract of *F. sycomorus* which showed antimicrobial activity against *E.coli* (14 mm), *S. aureus* (27 mm) and *C. albicans* (16 mm) but no antifungal activity to *A. niger* [16]. In addition, the methanolic and ethanolic leaf extracts showed inhibitory effect on test microorganism, *S. aureus*. [17]. Similar results were reported by Saleh and Al – Mariri (2017), who recorded that the acetone leaf extract of *F. sycomorus* showed good antibacterial activity against *Listeria monocytogenes, S. aureus, Bacillus cereus, Escherichia coli* O:157, *Salmonella typhimurium*, *Brucella melitensis, Proteus mirabilis*, *Yersinia enterocolitica* O:9, *Pseudomonas aeruginosa* and *Klebsiella pneumonia* [18]. This antibacterial activity on *S. aureus* (9 mm) was lower than the inhibition diameter of our leaf acetone extract (11 mm). However, in Braide et al's study no inhibition zone against *E.coli, Klebsiella spp.*, *S. aureus* and *P. aeruginosa* was observed in the leaf-methanolic extract [8].

In this study, inhibition against *C. albicans* and *S. aureus* at 100 mg/mL was observed. *S. aureus* causes superficial skin lesions (boils, shallots), localized abscesses, deep-seated infections, severe skin infections (furunculosis), infection of hospital-acquired surgical wounds and food poisoning. *C. albicans* causes fungal urinary tract infections (UTI), genital fungal infections, fungal skin infections and oral thrush. The susceptibility of *C. albicans* and *S. aureus* to leaf extracts of *F. sycomorus* was shown to be effective against diseases caused by these organisms and can be used as a healing agent.

In the study of Saleh et al., the MIC was evaluated by microdilution broth method to establish the susceptibility of pathogens to the acetone-leaf extract [9]. These values were found 7.3 mg/mL for resistant *S.aureus* and 6.6 mg/mL for sensitive *S. aureus*. In another study of Saleh and Al-Mariri, the MIC value of leaf-acetone extract against *S. aureus* was 130.2 mg/mL [18]. This value was rather higher than that of our acetone extract value (75 mg/mL.). Similar to our results, Jouda et al., showed that the MIC value was 25 mg/mL for leaf-ethanol extract against *S. aureus* [17]. Also, MIC value of leaf-methanolic extract against *S. aureus* was determined as 6.25-3.125 and 8.7-9.2 mg/mL in Saleh et al., and Jouda et al., studies which was lower than that of ours [9, 17].

The antioxidant activity and phytochemical properties of the *F. sycomorus* extracts were solvent dependent. In our study the total content of phenolic and flavonoid compounds varied in different fractions based on polarities of solvents ranging from 1.91± 0.33 to 11.29± 0.39 mg GAE/g; 0.07± 0.004 to 1.38± 0.021 mg RE/g (Table VII-VIII). The fruit extracts exhibited the highest DPPH removal activity followed by chloroform (69±1.21%), water (76±2.23%), acetone, ethanol and methanol (86±0.06-4.78%) (Table VII-VIII). This indicated that antioxidant activity was solvent – independent but was strongly dependent on the fruit samples taken from the *F. sycomorus*. The fruit extracts of *F. sycomorus* showed stronger antioxidant capacity as compared to leaf extracts.

Similarly, Al-matani et al., reported that the extractive yields of the *F. sycomorus* fruit varied depending on the polarity of solvents used [1]. The highest amount of total extractable compound was in the hexane extract (18.8%). This value was rather lower than that of our methanol extract (51.65%). Whereas, 11.09% and 4.06% the total extractable content value of methanolic and aqueous *F.sycomorus* leaf extracts reported by El Sayed et al., was higher than the values of this study [20].

Al-matani et al., predicated that TPC values of *F. sycomorus* fruit extracts were in the range of 3.43±2.16 and 81.56±0.43 mg GAE/100 g [1]. These were quite lower than the TPC values of *F. sycomorus* fruit extract calculated per g. El Sayed et al., noted as 124.00±4.96 and 26.31±3.76 mg GAE/g of the TPC of leaf methanol and water extract, which was dramatically higher than our results [20]. Moreover, we found out that in El-Beltagi et al's study., TFC values of *F. sycomorus* extracts (2.48±0.16 and 12.58±0.01 mg QE/ g) were much higher than our results [19].

This study supported previous reports by Samuel et al., and El Sayed et al. on the DPPH radical scavenging activity of F. *sycomorus* fruit and leaf extract prepared by different solvents [20, 21]. In our study, the ethanolic extract of *F. sycomorus* fruit gave the high antioxidant activity (86± 4.78%) as compared with the values reported (72.471%) by El-Beltagi et al. [19]. Antioxidant results of our study were in accordance with the results of Abdel-Aty et al., who found the highest value of DPPH radical scaveging activity (above 75%) in *F. sycomorus* latex extract [22].

In conclusion, *F. sycomorus* may be used as an alternative to antibiotics that are resistant to pathogenic microorganisms. In this study, leaf extracts were found to be more effective than fruit extracts against gram-positive *S. aureus* bacteria. It can also be used as medicine or food against infections caused by various bacteria or fungi. Fruits and leaves of *F. sycomorus* can be consumed as antioxidants against free radicals formed in the human body.

In the light of our findings, we think that *in-vivo* and *in-vitro* antioxidant mutagenic toxicity tests can guide the future studies on the effect of eukaryotic cells.

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Compliance with Ethical Standards

Authors' Contributions: Concept – M.S.O., F.T.; Design – M.S.O., F.T., H.A.M.T.; Supervision – M.S.O.; Resource – M.S.O., K.S., E.G., H.A.M.T; Materials – M.S.O., K.S., E.G., H.A.M.T; Data collection and/or Processing –M.S.O., F.T., E.G.; Analysis and/or interpretation – M.S.O., F.T., K.S., H.A.M.T.; Literature search – M.S.O., F.T.; Writing – M.S.O., F.T., H.A.M.T; Critical Reviews – M.S.O., K.S., H.A.M.T.

Conflict of Interest: The authors have no conflict of interest to declare.

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Anatomical variations detected during ultrasound-guided interscalene brachial plexus block and clinical implications

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ABSTRACT

Objective: Our aim was to evaluate the anatomic variations detected during ultrasound-guided interscalene brachial plexus block (US-ISB) and present their clinical implications.

Materials and Methods: After the ethical approval for the study was obtained from the local ethics committee, the files and US records of patients who underwent US-ISB for anesthesia of the shoulder surgery were retrospectively analyzed.

Results: Anatomical variations which were considered to affect the block technique were detected in 13 (11.8%) of 110 patients. C5 cervical root pierced the anterior scalene muscle (ASM) in 4.5%, and ventral rami of C5 and/or C6 were located in ASM in 3.6% of patients. There was a muscle bridge between C5 to C6 and C5 to C7 roots in 1.8% of the patients. The brachial plexus was located medial to ASM and missing from interscalene groove in 1.8% of patients. In one case (C5 root was located in ASM), US-ISB resulted in incomplete brachial plexus anesthesia, and so general anesthesia (GA) was performed.

Conclusion: Some of the brachial plexus variations in the interscalene area may be associated with further needle manipulation/redirection and block failure. We consider that prospective studies including more populations are needed to elucidate the effects of these variations on block parameters.

Keywords: Brachial plexus, Interscalene block, Ultrasonography, Variations

1. INTRODUCTION

The ultrasound-guided interscalene brachial plexus block (US-ISB) is one of the most common procedures for anesthesia and analgesia in the surgery of upper extremities. For the safe and effective regional anesthesia (RA) procedures, it is essential that all relevant anatomical structures must be properly defined. However, the brachial plexus has a complex anatomical structure, and the variations of the brachial plexus associated with its adjacent scalene muscles are common (11–49%) [1-5]. The variations usually occur as the course of the C5 and/or C6 cervical roots through or anterior to the anterior scalene muscle (ASM) [1,6]. Any of such variations can make it difficult to define the anatomical structures and cause confusion in practitioners with insufficient experience and familiar with the normal anatomy.

The variations associated with some nerves, muscles and vascular structures in the interscalene area may affect the technique of the procedure [7-9]. In the presence of such variations, especially in the cephalad approach and ISB conducted with low volume, the scalene muscles can prevent the spread of local anesthesia (LA) by acting as a barrier [10,11]. On the other hand, if high-volume LA is performed during ISB, unwanted phrenic nerve blockage and spreading to neighboring anatomical areas, such as epidural space, may occur.

The brachial plexus, needle, spreading of LA and surrounding anatomical structures can be visualized by US [12]. The variations in the interscalene area have been described in many studies; however, the number of the studies investigating the

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clinical effects of these variations is limited, and the majority are case reports.

In the present study, it was aimed to retrospectively evaluate the anatomic variations detected during US-ISB and present their clinical implications.

2. MATERIALS and METHODS

After the approval was obtained from the local ethics committee (2019/212) for the present descriptive retrospective study, the files and US records of the patients who underwent US-ISB for anesthesia of the shoulder surgery in a university hospital were analyzed between January 2019 and September 2019. The demographic data of the patients, such as age, gender and ASA the American Society of Anaesthesiologists (ASA) scores in the hospital archive were reviewed.

Interscalene Brachial Plexus Block

No US-ISB was performed in the patients with infections in the area of surgical intervention, psychiatric disease, neurological damage in the brachial plexus and respiratory failure, and those allergic to local anesthetics.

In our routine clinical practice, US-ISB is performed as described below. The block procedure is carried out by an anesthesiologist experienced in US-guided procedures or senior residents under the supervision of the same anesthesiologist.

While the patients are in the semilateral decubitus position, the 18 MHz linear probe (Esaote MyLab 30 Geneva, Italy) is placed in the supraclavicular fossa. After the brachial plexus is identified as a bright echogenic structure posterolateral to the subclavian artery (bunch of grapes sign), the probe is moved in the cephalic direction up to the interscalene groove by keeping the nerves in the center of the screen. The nerve structures are displayed as the string of hypoechoic circles (traffic light sign) between ASM and middle scalene muscles (MSM) (Figure 1). The sternocleidomastoid muscle, the common carotid artery and the internal jugular vein can be visualized as other main anatomical elements. Finally, the cervical roots are traced towards the cephalad until disappearing into the vertebra.

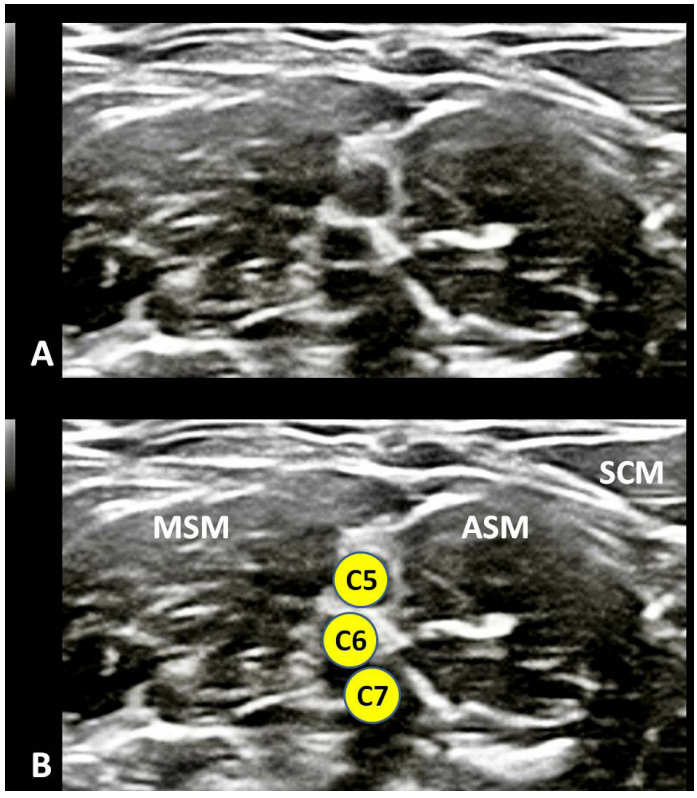

Figure 1. *A) Cross-sectional sonographic image of normal anatomy of the interscalene region, (B) illustrating nerve roots (yellow circles) in between the anterior scalene muscle (ASM) and middle scalene muscle (MSM). SCM: Sternocleidomastoid muscle*

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The needle tip is inserted using the in-plane technique and advanced through MSM under direct visualization into the interscalene space, and the nerves are confirmed by stimulating with the nerve stimulator. The spread of LA (20 mL, 0.25% bupivacaine) around the nerves is confirmed by US. The sensory and motor assessments are performed at 30th minute after the procedure. In our practice, the block was considered unsuccessful if any additional local analgesic or general anesthesia (GA) was used.

Although, the anatomic variations are often visualized during US scanning in the interscalene area, we can mostly identify a needle entry point where we can block the brachial plexus. In cases where the cervival roots are not visualized clearly in the interscalene groove, we perform the low interscalene block, supraclavicular block or GA. The US images of these variations of the brachial plexus are stored in the computer memory of US device for later analyses (Figure 2).

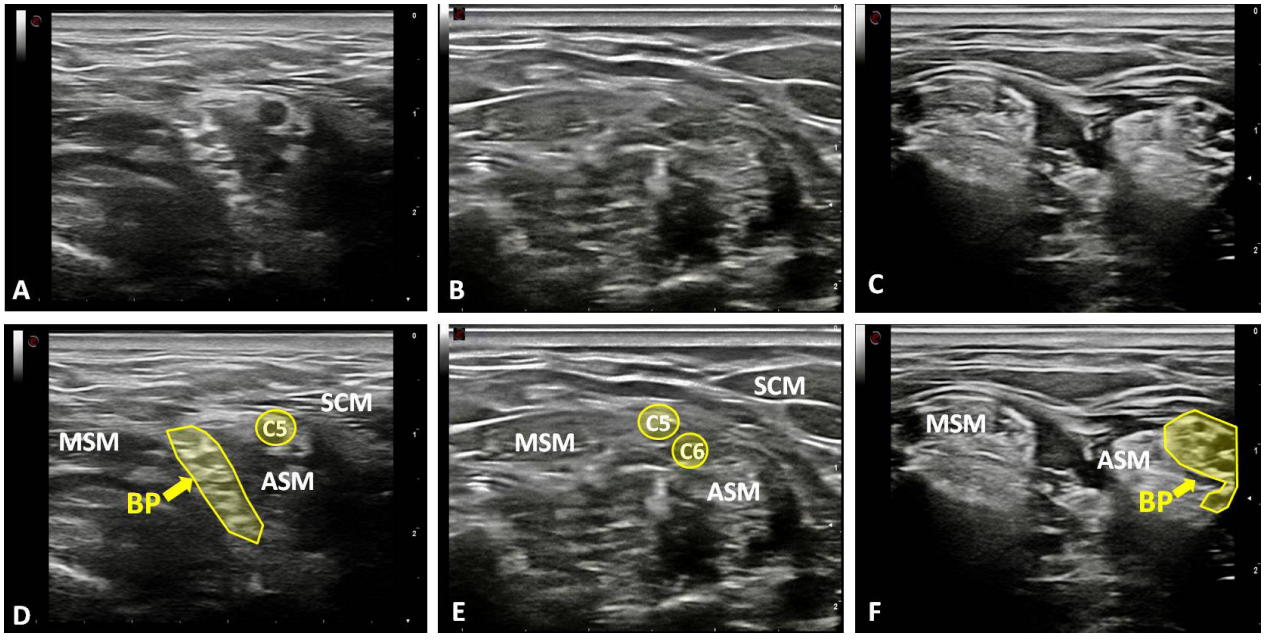

Figure 2. *Cross-sectional sonographic images of variations of the interscalene region in . (A,D) The ventral ramus of the C5 nerve root is located outside the interscalene groove, pierced the anterior scalene muscle (ASM), the brachial plexus (BP) is encircled by the yellow line. (B,E) The ventral rami of the C5 and C6 roots are located in ASM and a muscle bridge between scalene muscles. The C7 root is not visible in this plane. (C,F) BP (encircled by the yellow line) is located medial to ASM and missing from the interscalene groove.*

MSM: Middle scalene muscle, SCM: Sternocleidomastoid muscle

The recordings of these procedures and the data regarding US-ISB are re-evaluated by two experienced anesthesiologists. The patients with the shortage of data and administered with LA at different doses were excluded out of the study.

Statistical Analysis

The statistical analysis was performed using the Statistical Package of Social Sciences software for Windows, version 16.0 (SPSS Inc, Chicago, IL, USA). The descriptive statistics of the variables in the study were calculated, and while the continuous quantitative data were given as number, mean and standard deviation (SD) the qualitative data were expressed as number and percentage values. A p value <0.05 was considered to be significant.

3. RESULTS

A total of 119 RA records of the patients who underwent US-ISB were retrospectively reviewed, and nine patients were ruled out due to the shortage of data. The demographic and clinical data of the patients are presented in Table I.

The variations, considered to affect the block tecnique, were detected in 13 (11.8%) out of 110 patients who underwent US-ISB (Table II). While the ventral rami of C5 cervical spinal nerve pierced the ASM in 4.5% of the patients (Figure 1A), the ventral rami of C5 and/or C6 were located in ASM in 3.6% of the cases (Figure 1B). There was a muscle bridge between C5 to C6 and C5 to C7 roots in 1.8% of the patients. The brachial plexus was located at medial to ASM, and just lateral to the internal jugular vein (missing from interscalene groove) in 1.8% of the patients

(Figure 1C). In addition, the artery crossing or proximal to the roots was detected in 4.5% of the patients .

Table I. *Patients and clinical characteristics*

Value	mean±SD (n=110)
Age (years)	61.7±6.5
Gender (M/F) (%)	(%) 46 (42)/64 (58)
ASA I/II/III	19/64/27
Duration of surgery (min)	71.24 ±10.2
Type of surgery	
Rotator cuff repair	79 (71.9)
Bankart repair	16 (14.4)
Subacromial decompression	10 (9.1)
Capsular release	5 (4.6)

Data are given as number (proportion) or mean±standard deviation. ASA: The American Society of Anesthesiologists, SD: Standard deviation

Table II. *Anatomical variations detected during ultrasound-guided interscalene brachial plexus block*

Variations	Total, n=110 (%)
C5 pierce ASM	5 (4.5)
C5 and C6 pierce ASM together	4 (3.6)
Muscle bridge between roots	2 (1.8)
Ventral rami (C5-C7) located medial to ASM	2 (1.8)
An artery crossing roots	5 (4.5)

Data are given as number (proportion). ASM: Anterior scalene muscle

GA was performed in two cases where the brachial plexus was found to be located in the medial of ASM without any needle insertion. In one case, US-ISB resulted in incomplete brachial plexus anesthesia, and so GA was performed. In this case, C5 cervical root was localized in ASM, and the other cervical roots were not clearly visualized. The adequate paresthesia with motor blockade was achieved in all of 97 patients with normal anatomy. No intraoperative or postoperative complications, such as nerve damage, systemic LA toxicity or vascular puncture, were seen in any of the patients.

4. DISCUSSION

The main purpose of the present study was to evaluate the anatomical variations of the brachial plexus and adjacent structures in the interscalene area and to investigate their clinical significance. One of the most common variations we identified in the study is the dislocation of C5 ventral ramus out of the interscalene gap. The other is the muscular variations such as a muscle bridge between AMS and MSM separating the cervical roots from each other. In addition, the nerve structures of the entire brachial plexus were seen in the medial part of ASM in two of our patients.

In various studies, the incidence of the variations associated with the brachial plexus and ASM in the interscalene area was reported at different rates ranging between 11-49% [1-5]. In a study conducted by Harry et al. on cadavers, it was reported that the most common variation was that C5 and/or C6 ventral rami passed through or over ASM [1]. We observed C5 piercing anomalies in 4.5 % of our patients; even so, the rates of this variation were reported in the studies as 3.1% by Leonhard et al. [13] , 8% by Sakamoto et al. [14] and 13% by Harry et al [1] . However, the current study found a lower frequency of superior trunk piercing anomalies as 3.6%. As opposed to the percentage of 3.6% in our study, higher frequency of superior trunk piercing anomalies were stated as 15% and 39% in the studies by Harry et al. [1] and Leonhard et al. [13], respectively.

In the study performed by Kapral et al. the variations were detected in the interscalene region in 9 (11.2%) out of 80 cases [5]. In another study by Kessler et al., the variation of the brachial plexus was detected in 6 (13%) out of 46 patients (C5 ventral rami passed over the ASM in three patients and passed through ASM in three patients) [6].

In a case report by Chin et al., a patient with a C5 root located medial to the anterior scalene muscle (ASM) and a C6 root located in the inferomedial was reported [3]. In addition, in another case report performed by Yadav et al., most of the roots originating from the ventral rami were emphasized to be stacked medial to the ASM [15]. This variation, rarely reported so far, was encountered in two (1.8%) patients in our study. As different from previous cases, our patients would undergo shoulder surgery. Therefore, we performed GA for both cases.

In the study by Gutton et al., at least one variation was detected in the interscalene area through US in 71 (49%) out of 146 patients [4]. Of these 71 patients, while 8% had a C5 root anterior to ASM, 33% had an intramuscularly localized cervical root. Gutton et al. also reported that these variations had no relevant influence on the performance of ISB.

According to our results, however, US-ISB procedure failed in one of the patients determined with a variation. In some of the cases where the variations were visualized, additional needle insertion and more needle manipulation were required to ensure the adequate spread of LA. Anatomical variations may prolong the time of performing blocks. In addition, some variations also resulted in alterations in the anesthesia method.

The clinical effects of such variations become more evident when an anatomical landmark-guided technique is used for ISB. These variations may lead to the limitation of the distribution of injected LA and inadequate analgesia. The combination of anatomical variations may make the brachial plexus block difficult [7,8]. Therefore, the use of US becomes important and has a high success rate in the brachial plexus blocks developing with anatomical variations.

Since anatomical variations develop less in the supraclavicular region, the supraclavicular brachial plexus block may be chosen in the patients with variations, if the surgical method is appropriate.

The incomplete brachial plexus block may have occurred due to a number of reasons. We consider that the reason of the failed block is due to restriction of the spread of LA by ASM in this study.

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The anatomical variations may become more important, especially if ISB is performed in a more cephalad position and low volumes of LA solution. In our clinical practice, when these anomalies are observed, we perform ISB 2-3 cm caudal to the variation point, if possible.

Vascular structures, which will restrict the spread of LA or inhibit the needle advancement such as dorsal scapular artery, can be present between the cervical roots in this region [8,9]. While an artery crossing the roots or trunks was revealed in 23% of the patients in the study by Gutton et al., the rate was observed as 4.5% in the current study. Even if US is utilized, the practitioners should be more meticulous for intra-arterial injections [16]. The use of Doppler US will be beneficial to identify vessels.

The main limitations of our study are the retrospective nature and that all detailed information could not be available. In addition, the brachial plexus was unilaterally evaluated on the surgical side and we do not have data about the opposite side.

Although, the anatomic variations of the brachial plexus are frequently observed in the interscalene area by US, somehow the block procedure is mostly performed.

Here, the important issue is to differentiate the abnormal sonoanatomy. Some of the brachial plexus variations in the interscalene area may be associated with further needle manipulation/redirection and block failure. It may even be necessary using a different anesthesia technique, depending on the type of the variation. We consider that prospective studies including more populations are needed to elucidate the effects of these variations on block parameters.

Compliance with Ethical Standards

Ethical approval: The ethical approval was obtained from the local ethics committee (approval number: 2019/212)

Funding: The study was supported by no funds.

Conflict of interest: The authors have no conflicts of interest to declare.

Authors' Contibutions: AK designed the study, gathered and interpreted the data of the patients, performed the statistical analysis and drafted the manuscript. **FG** interpreted the data and revised the manuscript. **IHK** conceived the study and revised the manuscript. **AO and RY** gathered the data of the patients**.** All authors read and approved the final manuscript.

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The neuroprotective effect of lamotrigine against glutamate excitotoxicity in SH-SY5Y human neuroblastoma cells

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ABSTRACT

Objective: Glutamate-induced excitotoxicity has a role in the pathophysiology of neurodegenerative disorders. Lamotrigine, an antiepileptic drug, also used to treat bipolar disorders, may be protective against excitotoxic insult. The aim of the study was to investigate the neuroprotective effect of lamotrigine against the glutamate excitotoxicity in SH-SY5Y cell line.

Materials and Methods: SH-SY5Y human neuroblastoma cells were pre-treated with lamotrigine (50-100-150 μM) prior to exposure to 15 mM glutamate. The 3-(4,5-dimethythiazol – 2-yl)-2,5 – diphenyl tetrazolium bromide (MTT) assay was performed to determine cell viability. The anti-oxidant effect of lamotrigine and the role of inflammatory parameters were determined by measuring superoxide dismutase (SOD), hydrogen peroxide (H_2O_2), IL-1β, IL-6 and TNF-α.

Results: Intracellular calcium levels and lactate dehydrogenase (LDH) activity increased in glutamate exposed cells. Pre-treatment of cells with MK-801 showed no protective features against glutamate excitotoxicity. Treatment with 100 μM lamotrigine was effective in increasing the viability of glutamate exposed cells and in reducing H_2O_2 increase in these cells. The SOD activity increased by lamotrigine treated cells exposed to glutamate. IL-1β, IL-6 and TNF-α levels increased after induction with glutamate and attenuated by lamotrigine.

Conclusion: Overall, our results confirmed the critical role of inflammation and oxidative stress in glutamate-induced excitotoxicity and lamotrigine may exert a protective effect.

Keywords: Lamotrigine, Glutamate excitotoxicity, SH-SY5Y, Oxidative stress parameter, Cytokines, MK-801

1. INTRODUCTION

Lamotrigine is an anticonvulsant drug used in the treatment of bipolar disorder. It has a modest effect in depressive episodes and its benefit in maintenance therapy is more effective when combined with other mood stabilizers such as lithium or valproate [1,2].

As, lamotrigine has a broad anticonvulsant spectrum and psychotropic profile, it is proposed to have distinctive cellular effects that contribute to its broad effects. Its well-demonstrated cellular mechanism of action is the blockade of neuronal voltage-gated sodium channels and subsequent stabilization of neuronal membranes and suppression of post-synaptic glutamate release [3].

The disruption of normal excitatory neurotransmission regulated by glutamate and its ligand-gated ionotropic glutamate receptors are involved in a wide range of pathophysiology of neurological conditions such as epilepsy [4], hypoxic-ischemic brain damage [5] and neurodegenerative disorders such as Alzheimer's, Parkinson's, Huntington's disease and multiple sclerosis [6].

The involvement of abnormal Na^+ influx and voltage-gated sodium channel activity in the pathophysiology of these disorders lead to the investigation of neuroprotective effects of lamotrigine. Hence, it is effective in status epilepticus [7], oxygen-glucose deprivation [8], neonatal hypoxic-ischemia [9] and adult ischemia [10]. Despite its widespread clinical use and accepted neuroprotective effects, the molecular mechanisms of its therapeutic actions need to be identified.

Chronic neuroinflammation plays an important role in the pathogenesis of neurodegenerative disorders, such as Alzheimer's disease, Parkinson's disease and multiple sclerosis [11]. It is also known that these diseases develop in older age

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and normal aging process induces several cellular changes including increase in intracellular Ca^{2+} levels which causes low-grade inflammation in the central nervous system (CNS) and the peripheral systems [12]. This low-grade inflammation is associated with increase in inflammatory mediator release such as interleukin-1beta (IL-1β), interleukin-6 (IL-6) and tumor necrosis factor-alpha (TNF-α).

Thus, regulating pro-inflammatory mediators in neurodegenerative disorders may have the therapeutic potential to reduce neuronal injury in neurodegenerative diseases. Infection or inflammation that occurs in response to injury, is associated with several neurotoxic and pro-inflammatory mediators. These mediators are reactive oxygen species (ROS), nitric oxide, prostaglandin E_2, as well as pro-inflammatory cytokines such as IL-1β, IL-6 and TNF-α. Accordingly, we investigated the neuroprotective effect of lamotrigine against glutamate toxicity in the SH-SY5Y cell line by measuring inflammatory and oxidative system parameters.

2. MATERIALS and METHODS

Cell culture model

The study was conducted using the SH-SY5Y human neuroblastoma cell line. The SH-SY5Y cells were grown in Dulbecco's Modified Eagle's Medium (Thermo Fisher Scientific Inc., UK) supplemented with 10% fetal bovine serum (Thermo Fisher Scientific Inc., UK), 100 IU/ml penicillin and 100 µg/ml streptomycin (Thermo Fisher Scientific Inc., UK). The cells were seeded into 96-well plates ($1x10^4$ cells/well) and cultured in an atmosphere of 5% CO_2 and saturated humidity at 37.0˚C. SH-SY5Y cells were incubated in complete culture medium for 24 h prior to the addition of glutamate or lamotrigine regarding the investigation of the effects of glutamate and lamotrigine.

Drug concentrations

Cells were treated with different concentrations of glutamate (1-50 mM; L-Glutamic acid; cat. no. G1251; Sigma-Aldrich, USA) in order to determine the glutamate toxicity in the cultured SH-SY5Y cells. 15 mM glutamate produced a significant decrease in cell viability, ~20% of control after 24 h. Subsequently, 15 mM was selected as the working concentration of glutamate to be used in the following experiments.

MK-801 was purchased from Sigma-Aldrich Co. (cat. No. M107, Germany) and used to investigate the role of N-methyl-D-aspartate (NMDA) receptor in glutamate toxicity. MK-801 (1, 5, or 10 µM) was added to culture medium 3 h before and co-incubated with 15 mM glutamate for 24 h.

The SH-SY5Y cells were treated with lamotrigine (Sigma-Aldrich Co. cat. No. L3791, Germany), concentrations of 50 µM, 100 µM, 150 µM 1 h prior to glutamate exposure. Lamotrigine was dissolved in 12 mg / ml dimethyl sulfoxide. As 100 µM was found to be the neuroprotective concentration of lamotrigine, this concentration was used in the following experiments.

Cell viability assay

A 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay kit (Thermo Fisher Scientific Inc., USA) was used to evaluate cell viability in line with the manufacturer's instructions. After adding MTT solution (5 mg/ml) to each well, cells were incubated for 3 h with 5% CO_2 at 37˚C. Following the removal of the culture medium, 200 µl dimethyl sulfoxide was used to dissolve the formazan product. Absorbance values were measured at 560 nm wavelength using a microplate reader (Multiskan™ GO microplate spectrophotometer; Thermo Fisher Scientific Inc., Finland). Cell viability was calculated by considering the controls as 100%.

Cell lysate preparation

SH-SY5Y cells were harvested by using Trypsin-EDTA 0.25% (Thermo Fisher Scientific Inc., UK) and collected by centrifugation at 1,000-2,000 x g for 10 min at 4˚C. Onwards, cells were then re-suspended with ice-cold buffer (0.05 M potassium phosphate, pH 7.0, 1 mM EDTA) then homogenized by sonication on ice. The lysate was centrifuged at 12,000 x g at 4˚C for 20 min to remove cell debris. The supernatant was further used for determining the quantity of total protein and for the enzyme activity assay. The protein concentration was determined using the bicinchoninic acid assay kit (Thermo Fisher Scientific Inc., USA). All spectrophotometric measurements were made using an Epoch microplate spectrophotometer (BioTek Instruments, Inc., USA).

Intracellular calcium concentration ([Ca2]i) assay

Intracellular Ca^{+2} measurement was performed using Ca^{+2} colorimetric assay kit (CAT No:K380; Biovision Inc., USA). The kit utilizes the chromogenic complex (λ = 575 nm) formed between calcium ions and 0-cresolphthalein and calcium concentration determined by means of a standard curve generated using calcium standard (500 mM). All samples, standards and controls were measured in duplicate.

Lactate dehydrogenase (LDH) assay

The LDH cytotoxicity was determined in the prepared supernatant with an enzyme linked immunosorbent assay (ELISA) kit (Pierce LDH Cytotoxicity Assay Kit; Thermo Fisher Scientific Inc., USA) according to the manufacturer's instructions.

Cytotoxicity was calculated with the following equation;

Cytotoxicity (%)=(Sample LDH activity – Spontaneous LDH activity)/(Maximum LDH activity – Spontaneous LDH activity) x 100

IL-1β, IL-6, TNF-α assay

The concentrations of IL-1β, IL-6 and TNF-α were determined using ELISA kits (Invitrogen BMS224HS, KHC0061, BMS223HS; Austria). According to the manufacturer's instructions, a colored product was formed in proportion to the amount of cytokines present in the sample or standard. The reaction was terminated by

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addition of acid and absorbance was measured at 450 nm. A standard curve was prepared from standard dilutions for each cytokine and sample concentration was determined. All of the samples, standards and controls were estimated using duplicate analyses.

Superoxide dismutase (SOD) activity assay.

The SOD assay was performed by quantifying the inhibition of nitro blue tetrazolium (NBT) at 560 nm wavelength. The assay mixture (200 µl) comprised of 0.0033 mM riboflavin, 10 mM L-methionine, 0.033 mM NBT and 0.66 mM EDTA-Na_2 in 0.05 M potassium phosphate buffer (pH 7.8). The 96-well plates containing the assay mixture were incubated for 20 min with 300 nmol/m^2/sec at 560 nm excitation at 25˚C. One unit of SOD activity was defined as the amount of protein (mg) causing 50% inhibition of photoreduction, following which specific enzyme activity was expressed as units/mg protein.

Hydrogen peroxide (H_2O_2) activity assay

The H_2O_2 activity in cells were quantified using H_2O_2 assay kit (cat no. ab102500; Abcam, USA). At the 24th h after drug administration, cells were harvested, homogenized and centrifuged. The supernatant was used for the assay. The absorbance was detected at 570 nm using a microplate reader and the optical density was used for quantification of H_2O_2 levels. Distilled water was used as a negative control instead of a cell lysate sample.

Statistical Analysis

Values are expressed as the mean ± standard error of the mean and analyzed by one-way analysis of variance (ANOVA) followed by a Tukey's multiple-comparisons post-hoc test. A P value <0.05 was regarded as a statistically significant difference.

3. RESULTS

There was a significant decrease in cell viability in 15 mM glutamate exposed cells without any treatment (Figures 1 and 2). As shown in Figure 1, MK-801 pretreatment in three different doses have not protected cells from glutamate-induced excitotoxicity. MK-801 alone did not exhibit any significant effect on the cell's activity.

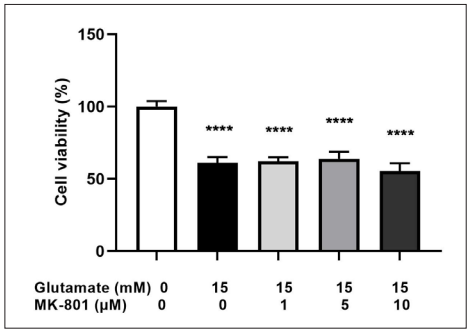

Figure 1. *Figure indicating the 15 mM glutamate-induced decrease in cell viability in SH-SY5Y cells as % of control. Cells were treated with 3 different concentrations of MK-801 (1, 5, or 10 µM). Cell viability (% of control) is expressed as the mean value of four separate experiments. ****P<0.0001 vs. control*

After cells were treated with lamotrigine, the cell viability was enhanced, and the protective effect of lamotrigine on cells after glutamate exposure was significant at a concentration of 100 µM (Figure 2).

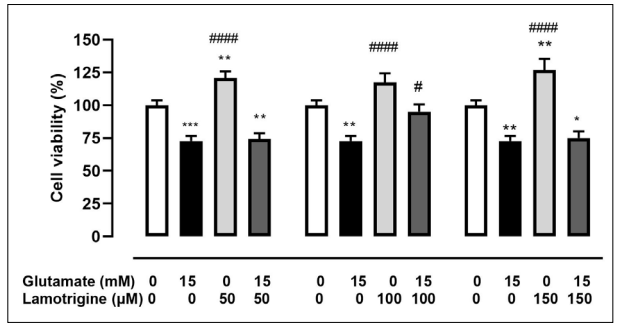

Figure 2. *Effect of lamotrigine pre-treatment on the viability of SH-SY5Y cells with glutamate-induced excitotoxicity. Viability of SH-SY5Y cells pre-treated with or without three different lamotrigine concentrations (50, 100, 150 µM) followed by exposure to 15 mM glutamate to induce excitotoxicity. Cell viability (% of control) is expressed as the mean value of four separate experiments. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001 vs control group; # P<0.05, #### P<0.0001 vs glutamate group*

Intracellular calcium levels in glutamate exposed cells were detected to be increased when compared to control cells that were not exposed to glutamate (P<0.05 vs control group). Lamotrigine significantly inhibited glutamate-induced elevation of [Ca^{2+}]I (P<0.005 vs glutamate group; Figure 3A).

The LDH activity increased with glutamate cytotoxicity and decreased significantly below the control levels in lamotrigine pre-treated cells that were exposed to glutamate (P<0.05; P<0.005 vs control group; Figure 3B).

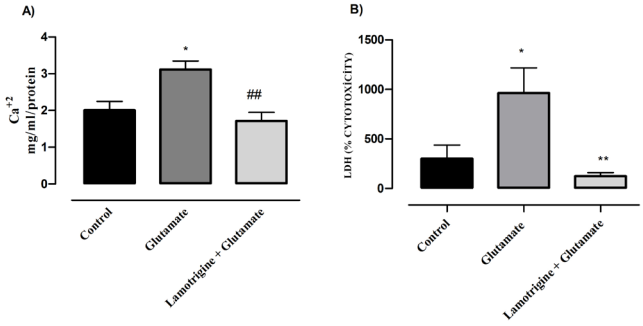

Figure 3. *(A) Ca^{2+} and (B) LDH levels in lamotrigine-pre-treated SH-SY5Y cells exposed to glutamate to induce excitotoxicity. *P<0.05 vs. control group; ##P<0.01 vs. glutamate group in A. *P<0.05, **P<0.01 vs. control group in B. Values are expressed as the mean value of four separate experiments.*

The IL-1β, IL-6 and TNF-α levels increased significantly after induction with glutamate. Lamotrigine application attenuated these increased levels induced by glutamate (Figure 4).

The SOD activity slightly increased after glutamate exposure, but this alteration was not statistically significant. The SOD

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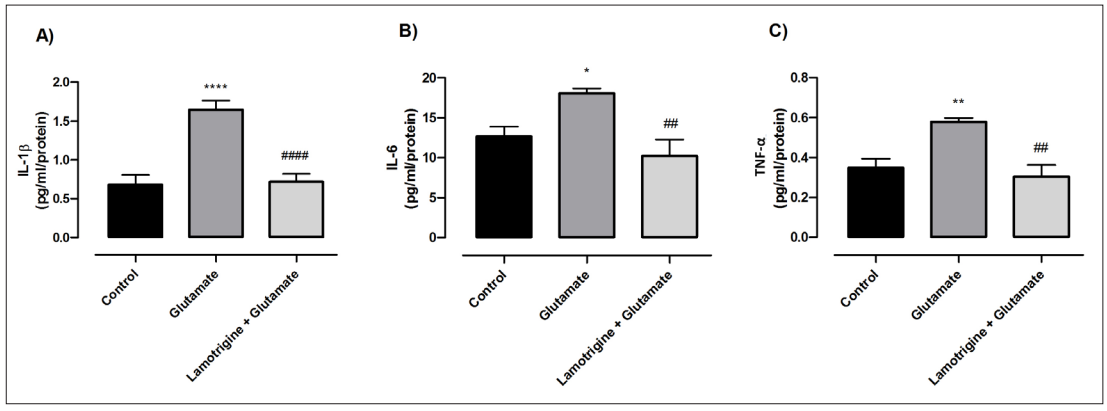

Figure 4. *(A) Interleukin-1β (IL-1β), (B) Interleukin-6 (IL-6) and (C) Tumor necrosis factor – α (TNF – α) levels in lamotrigine-treated cells exposed to glutamate. ****P<0.0001 vs control group; #### P<0.0001 vs glutamate group in A. *P<0.05 vs. control; ##P<0.01 vs. glutamate group in B; **P<0.01 vs control group; ## P<0.01 vs glutamate group in C.*

activity increased in the lamotrigine pre-treated cells when exposed to glutamate. This increase was significant as compared with that in the control group and glutamate group (P<0.001; P<0.01, respectively; Figure 5A).

After 24 h of incubation with 15 mM glutamate, H_2O_2 levels increased as compared with those in the control group (P<0.0001). Treatment with lamotrigine was indicated to decrease H_2O_2 levels in cells exposed to glutamate when compared to treatment with glutamate alone (P<0.0001), and data revealed that it was able to reduce it to the control levels (Figure 5B).

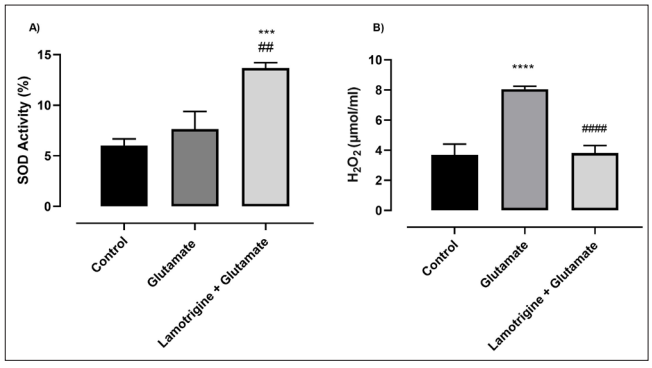

Figure 5. *(A) SOD activity and (B) H_2O_2 in control cells, lamotrigine-treated cells, cells exposed to glutamate, as well as lamotrigine-pre-treated SH-SY5Y cells exposed to glutamate excitotoxicity. ***P<0.001 vs control group; ## P<0.01 vs glutamate group in A; ****P<0.0001 vs control group; #### P<0.0001 vs glutamate group in B. SOD, superoxide dismutase; H_2O_2, hydrogen peroxide*

4. DISCUSSION

Cell viability analysis was used as an indication of cell death to determine the toxic glutamate dose for SH-SY5Y cell lines. We demonstrated that pre-treatment with 100 μM lamotrigine significantly inhibited cell toxicity caused by glutamate as a measurement of cell viability. The results of cytotoxicity due to LDH release also supported this finding. The neuroprotective effect of lamotrigine was investigated in several in-vivo models and it was shown that 10 and 20 mg/kg lamotrigine had protective effect in vivo model of neonatal hypoxic-ischemic encephalopathy in rats [9]. In the study of Halonen et.al., 12.5 mg/kg, twice a day lamotrigine treatment demonstrated reduction in the duration or severity of the status epilepticus, showing mild neuroprotective effect observed in the hippocampus and piriform cortex of rats [7]. Walker et al., reported that the mean serum concentration was 23.03±2.1 μM with 10 mg/kg and 46.6 ±7.7 μM with 20 mg/kg administration of lamotrigine [13]. However, similar to our results, Leng et al., revealed that 100 μM lamotrigine had a neuroprotective effect in glutamate-induced primary neuronal cerebellar granule cells via histone deacetylases (HDAC) inhibition and up-regulation of anti-apoptotic Bcl-2 [14]. Also, the dose used in the present study was comparable to the lamotrigine's therapeutic target range of 10–60 μM in the serum [15].

We found that MK-801, a NMDA receptor antagonist was not effective in reversing cell viability which was decreased by glutamate administration suggesting that the cytotoxic effect of glutamate in SH-SY5Y cells was related with any other mechanism rather than NMDA receptor. The previous studies showed controversial results that the effect of glutamate on SH-SY5Y cells was whether NMDA-mediated or not [16-19]. The expression of metabotropic and ionotropic receptors was shown in this cell line by some studies [16,17] whereas, others revealed that NMDA receptors were not expressed in SH-SY5Y cells, suggesting the increase of cytoplasmic Ca^{+2} was independent of glutamate receptors [18,19]. Glutamate toxicity could be explained in two forms, one was receptor-related excitotoxicity [20] and the other was non-receptor related oxidative glutamate toxicity [21]. It was reported that after glutamate exposure, calcium influx caused induction of free radical generation from mitochondria and these free radicals could cause cell membrane peroxidation and might activate inflammation signaling pathways with cell damage, and might also disrupt

the blood–brain barrier by affecting the endothelial basement membrane [22].

We determined that intracellular calcium levels increased in glutamate exposed cells. This finding was in accordance with the other reports revealing that the Ca^{2+} dependent release of glutamate involved intracellular Ca^{2+} stores in astrocytes [23,24]. It is speculated that sustained Ca^{2+} influx through glutamate receptor channels was an important pathway of neuronal cell death. The increase of glutamate levels in the CNS might cause elevated intracellular Ca^{2+} levels, which lead to a rise in the Ca^{2+} concentration in sensitive organelles such as mitochondria and the endoplasmic reticulum [25]. It was accepted that the sustained high levels of intracellular Ca^{2+} subsequent to Na^{+} or both, might lead to neuronal degeneration involving several different pathways that caused oxidative stress and degeneration [21,26]. We demonstrated that lamotrigine decreased the elevated Ca^{2+} levels due to glutamate exposure. In accordance with this finding, it was reported that lamotrigine, besides its action on voltage-dependent sodium channels, also affected the neuronal calcium channel, and calcium antagonistic actions were discussed to have a role in treatment strategies of epilepsies [27].

Glutamate, a major excitatory neurotransmitter in CNS, may lead to the development of various neurodegenerative diseases when present at high concentrations by inducing oxidative stress and neurotoxicity. Oxidative stress has an important role leading to neuronal loss and death. Glutamate-induced cell death involves the inhibition of glutathione synthesis and depletion and causes excessive ROS production resulting in oxidative stress. The accumulation of excessive ROS can cause functional and structural changes in the mitochondria and activate cell death pathways [21,28].

We evaluated the levels of SOD and H_2O_2 to determine the role of oxidative stress on glutamate-induced cell death in SH-SY5Y cells. H_2O_2, can readily cross the cell membrane, affects cellular structure distant from its origin and is considered most suitable for redox signaling among the various oxygen metabolites [29]. We found a significant increase in H_2O_2 levels after acute exposure of 15 mM glutamate in SH-SY5Y neuroblastoma cells suggesting that glutamate-induced excitotoxicity was mediated with oxidative stress. The studies suggesting the contribution of oxidative stress in glutamate excitotoxicity also presented similar findings with our study. Ha et al., stated an accumulation of extracellular H_2O_2 after prolonged exposure to glutamate in a time – and concentration-dependent manner in HT22 cells [30]. It was reported that glutamate at 1-50 mM concentration affected H_2O_2 synthesis by brain mitochondria and this effect was associated with complex II, a source of superoxide anion formation in mitochondria and was dependent on the mitochondrial potential [31]. We found a non-significant increase in SOD activity which was not consistent with other studies that had reported a decrease with glutamate [18,32]. ROS is converted to less reactive hydrogen peroxide by SOD through the use of copper/zinc or manganese and could play a protective role in neurodegeneration and had been thought to be activated firstly for defense systems against oxidative stress, in neurodegeneration [33]. We think that a protective mechanism was activated as a response to increased ROS after glutamate exposure as mentioned that increase of SOD was related to the adaptive mechanism against the raised amount of lead-induced ROS production [34].

We also determined that pre-treatment with lamotrigine caused an increase in SOD activity whereas a decrease in H_2O_2 levels when compared to the only glutamate exposed SH-SY5Y cells. So, it could be speculated that the protective effect of lamotrigine against glutamate toxicity was related to its antioxidant activity. In the study of Kamal et al., it was revealed that the management of epilepsy by lamotrigine could be associated with the possible antioxidant activity, supporting our data [35]. Moreover, in a study evaluating the protective effects of lamotrigine, aripiprazole and escitalopram on depression-induced oxidative stress it was found that lamotrigine had the most protective effect on the oxidative stress within the three drugs [36].

We determined an increase in TNF-α and also in IL-1β, IL-6 levels after induction of 15 mM glutamate in SH-SY5Y cells, and this increase was attenuated by lamotrigine significantly. Similar to our study, in an Alzheimer's model on SH-SY5Y cells it was shown that TNF-α levels increased following treatment with Amyloid-β (Aβ) [37]. Both TNF-α and IL-6 levels elevated in the mechanical trauma injury-induced SH-SY5Y cell model [38]. The levels of inflammatory factors increased in nerve cells under pathological conditions such as ischemia, hypoxemia, mitogens, cytokines and hormones leading to neuronal degeneration [39]. Glial cells including astrocytes and microglia are generally accepted as the major sources of pro-inflammatory cytokine production in the CNS. However, it has been reported that both glial and neuronal cells can produce and release pro-inflammatory cytokines with interaction with chemokines, and adhesion molecules in response to toxic stimuli. Proinflammatory cytokines modulate inflammatory processes [40].

The findings of this study demonstrated a significant reduction of IL-1β, IL-6 and TNF-α levels with lamotrigine in the glutamate-exposed cells. The results of some other studies showed differences in the effects of lamotrigine on cytokine levels. Similar to our findings, it was reported that lamotrigine caused a consistent reduction in IL-6 and TNF-α secretion both in vivo and in vitro lipopolysaccharide (LPS)/concanavalin A (ConA)-induced inflammation model, whereas, only in ConA-induced inflammation model for IL-1β [41,42]. An inhibitory in vitro effect of lamotrigine on TNF-α and IL-1β was seen, whereas, no effect on IL-6 secretion was reported after stimulation of whole blood obtained from healthy female subjects, by toxic shock syndrome toxin-1 [43]. Additionally, IL-1β secretion decreased by lamotrigine with no change on TNF-α and IL-6 levels by stimulation with a combination of anti-CD3 and anti-CD40 antibodies. It was speculated that the variability effects on the proinflammatory cytokines among the studies could be due to the differences in the stimulants, cells and other experimental settings [44].

In conclusion, the results of the present study demonstrate that lamotrigine exerts neuroprotective effects against glutamate

induced toxicity in SH-SY5Y cells by reducing oxidative stress through increased levels of antioxidant enzymes and the proinflammatory cytokines.

Compliance with Ethical Standards

Ethical approval: According to the Institutional Ethical Committee, this study did not require ethics approval as it was conducted on cell lines and the data did not contain patient-specific information.

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Author Contributions:

Concept and Design – BTB, EO, NGA, AH; Supervision – BTB, EO, NGA; Resources – BTB, EO, NGA, AH; Materials – BTB, EO, NGA, AH; Data Collection and Processing – BTB, EO, NGA, AH, FT, SA, AK; Analysis and Interpretation – BTB, EO, NGA, AH, FT, SA, AK; Literature Search – BTB, EO, NGA, AH, FT, SA, AK; Writing Manuscript – BTB, EO, NGA, AH, FT, SA, AK; Critical Review – BTB, EO, NGA, AH, FT, SA, AK

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Identification of cleaning staff's habits of personal hygiene and evaluation of the effectiveness of the training carried out

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ABSTRACT

Objective: In this research, in which the effectiveness of the training given to the cleaning staff who are likely to be carriers for pathogens was investigated, it was also examined whether the socio-demographic characteristics of the participants lead to any difference in their hygiene practices.

Materials and Methods: Training on hygiene was given to the participants by a physician who is specialized in the field of infection. Their knowledge before and after the training was evaluated.

Results: It was determined that the rate of desired responses in correct practices regarding hygiene was low, and that there was a change in the scores related to some areas (frequency of going to the dentist, wrong practices in hair hygiene, face towel, hand cleaning material, foot towel practices) after training.

Conclusion: In the research, the effect of health training provided to cleaning staff on knowledge and behavior was examined. As a result of the research, it was observed that there was a general positive increase related to the hygiene issues in the level of knowledge and behavior of the cleaning staff.

Keywords: Cleaning staff, Hygiene, Before and after training

1. INTRODUCTION

According to the World Health Organization (WHO), hygiene is the total of conditions and practices for protecting health and preventing the spread of diseases [1].

Personal hygiene is called personal care practices that enable individuals to protect and maintain their health. Personal care, on the other hand, is the whole of activities that are initiated and implemented by the individual aimed at maintaining life, health and well-being. It is the implementation of the practices necessary for individuals' own health by the individuals themselves instead of expecting others to implement them on their behalf [2].

In today's societies where personal hygiene is very important, it is possible to provide a happier, more peaceful and successful education system by taking all necessary precautions for the continuation of a healthy life in public life areas that are closely related to human health. In order to achieve this goal, attention should be paid to the interaction between students at the center of the education system, university and environment, and efforts should be made to create a healthier environment by acting in accordance with personal hygiene and health practices as much as possible. When the literature is analyzed, it is seen that the studies conducted on health and hygiene education in the institutions providing education focus mostly on determining the health knowledge levels and health behaviors of the students.

In studies conducted on personal hygiene in all educational institutions, it was determined that the correct hand washing rate among students was low and that the physical environment of educational institutions should have a setting that lends itself to cleaning [3,4].One of the most important parts of this environment is undoubtedly the cleaning staff working in educational institutions. For this reason, considering that it can be important in terms of school health in all educational institutions and beneficial results would be obtained, in this study, it was aimed to determine the personal hygiene habits of cleaning staff working in a university and to evaluate the effectiveness of the training activity carried out.

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2. MATERIAL and METHODS

The research had a quantitative research design with a semi-experimental type including before-training and after-training comparison. The data were collected between June and December 2019. The study had a relational design as it relied on the measurements of the participants related to the dependent variable before and after the procedure. The population of the research consisted of 97 cleaning staff working in a state university in a provincial center, and the sample of the study consisted of cleaning staff (n=94) working at the university during the data collection process who responded to the questionnaire before and after the training. Some trainings were explained theoretically within the scope of its content, and some trainings were delivered as practical training (hand hygiene, tooth brushing, eye cleaning, nail cutting, etc). The participation rate in the research was 96.9%.

The before-training test was applied to the participants in the meeting room in 40 minutes under the control of one of the research assistants. In order to obtain objective data in the test, codes were assigned to the questionnaire forms of the participants, and in order for these codes to be remembered, the identity information and codes of the participants were listed. The list was entrusted to the head of the cleaning staff who did not participate in the study, and the participants were asked to memorize their codes. Then, the participants were divided into groups of 20, and each group was provided with face-to-face training by a specialist physician in infectious diseases. The after-training test was administered by the researchers through face-to-face interview method three months after the training. Fourteen participants who forgot their codes learned their codes from the person who had the code list. The list was then destroyed. All procedures performed in the current study were in accordance with the ethical standards of the institutional ethics committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. This study was approved by Bingöl University Ethics Committee (Approval date and number: 15.04.2019; 92342550/044 – E.8055). Before the data for the study started to be collected, the participants were informed that they could withdraw from the study in accordance with the principle of 'Autonomy' for the purpose of the protection of participants' rights, and the ''Informed Consent Form" was presented to them. The principle of 'Confidentiality' was respected during the study; it was ensured that the identity of the participant and the data obtained were kept confidential. The questionnaires belonging to the study were distributed to and collected from the participants via a research assistant in order not to influence the voluntariness of the participants and direction of the research. In line with the principle of 'Respect for Human Dignity', the participants were not judged because of their opinions and practices.

Statistical Analyses

The socio-demographic characteristics of the participants constituted the independent variables of the research, and questions about personal hygiene were determined as the dependent variables. Statistical Package for the Social Sciences-22 (SPSS-22) was used for the analyses; error checks were run and tables were created through the program. Descriptive data were presented in numbers and percentages. Mc Nemar-Bowker analysis was used to analyze the data. The averages were provided with standard deviations, $P<0.05$ was accepted as the significance level.

3. RESULTS

The participants (50.0%) in the research were between 36-45 years old, and 27.7% of the participants were females. The average working year of the participants was 8.46 ± 4.94 (median:8, min:1, max:29 years), and the average working year as cleaning staff was 6.71 ± 3.03 (median:7, min:2, max:15 years) (Table I).

Table I. *Descriptive characteristics of the participants (N = 94)*

Characteristics	Number	%
Age group		
Under 25 years of age	5	5.3
Between 26-35 years	26	27.7
Between 36-45 years	47	50.0
Above 46 years of age	16	17.0
Gender		
Female	26	27.7
Male	28	72.3
Marital status		
Married	81	86.2
Single	13	13.8
Educational level		
Illiterate	4	4.3
Literate	2	2.1
Primary school	38	40.4
Secondary school	20	21.3
High school	25	26.6
University	5	5.3
Total working years		
Less than 5 years	24	25.5
Between 6 and 10 years	55	58.5
11 years and above	15	16.0
Working year as a cleaning staff		
Less than 5 years	39	41.5
Between 6 and 10 years	48	51.1
11 years and above	7	7.4

As seen in Table II, in terms of before-training and after-training, it was determined that the variables other than the dependent variables of the frequency of going to the dentist ($P= 0.005$), the knowledge related to the wrong practice of hair cleaning ($P= 0.046$), personal or common use of face towel ($P = 0.002$), foot towel ($P = 0.001$) and hand soap ($P = 0.041$) did not display any difference ($P > 0.05$). After the training, an increase by 10% in the rate of those who said they should visit the dentist in 3 months or less was determined. Regarding the wrong practice related to hair cleaning, participants answered that "The hair should be brushed regularly in order to remove dirt and dead hair" in the group after the training with a rate of

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more than 10% increase. The rate of increase in those who said that oily hair should be washed more frequently was around 8% in the training group. It was determined that the after-training group stated that the face towel should belong to the person with a 5% increase. On the other hand, following the training, it was determined that the training group stated that the foot towel should belong to the person with an increase of approximately 18%. Those who stated that hand soap should belong to the person were found to increase by 10% in the group after the training.

Table II. *Status of participants' hygiene behaviors before and after training (n = 94)*

Hygiene Behavior	BT n (%)**	AT n (%)**	Test Value*
The effects of general body cleanliness			
It enables the odor of the body to be expelled.	7 (7.4)	13 (13.8)	
It relieves the individual psychologically.	11 (11.7)	11 (11.7)	p=0.573
It removes some microorganisms from the skin.	15 (16.0)	14 (14.9)	
It causes the excess fat in the body to be burnt.	61 (64.9)	56 (59.6)	
Frequency and form of body cleaning			
The body needs to be washed with soap and rinsed every two weeks.	13 (13.8)	11 (11.7)	
The body needs to be washed with soap and rinsed every day	41 (43.6)	44 (46.8)	p=0.170
The body needs to be washed with soap and rinsed once a week	9 (9.6)	19 (20.2)	
Taking a shower without soap is necessary every morning.	31 (33.0)	20 (21.3)	
Which one do you apply to remove odors such as sweat other than having a bath?			
I use deodorant.	33 (35.1)	30 (31.9)	
I wash my body parts such as armpits with soap and water.	25 (26.6)	16 (17.0)	p=0.209
I change my underwear every day.	29 (30.9)	36 (38.3)	
I wipe my body parts such as armpits with a soapy washrag.	4 (4.3)	6 (6.4)	
Other	3 (3.2)	6 (6.4)	
How do you clean your face?			
I wash my face with water in the morning and at night before going to bed.	33 (35.1)	26 (27.7)	
I wash it with water and suitable soap in the morning and at night.	17 (18.1)	24 (25.5)	p=0.506
I wash it with soap and water in the morning, at night before bedtime and during the day.	40 (42.6)	40 (42.6)	
Other	4 (4.3)	4 (4.3)	
Do you have your personal towel at work?			
Yes	52 (55.3)	57 (60.6)	p=0.359
No	42 (44.7)	37 (39.4)	
Do you also wash your hair separately apart from taking a bath?			
Yes	81 (86.2)	84 (89.4)	p=0.453
No	13 (13.8)	10 (10.6)	
How often do you visit the dentist?			
Never	20 (21.3)	16 (17.0)	
In three months or less	17 (18.1)	27 (28.7)	**p=0.005**
Once in six months	34 (36.2)	28 (29.8)	
In a year or more	23 (24.5)	23 (24.5)	
How often do you change your toothbrush?			
In three months or less	64 (68.8)	63 (67.7)	
Once in six months	17 (18.3)	21 (22.6)	p=0.240
In a year or more	7 (7.5)	8 (8.6)	
Other	5 (5.4)	1 (1.1)	
Do you regularly brush your teeth?			
Yes	82 (87.2)	84 (89.4)	p=0.687
No	12 (12.8)	10 (10.6)	
Why should teeth be brushed regularly?			
For health	54 (57.4)	58 (61.7)	
For cleanliness	5 (5.3)	7 (7.4)	p=0.269
To prevent decay	31 (33.0)	26 (27.7)	
Against bad breath	2 (2.1)	1 (1.1)	
Other	2 (2.1)	2 (2.1)	
Which one is the wrong practice about hair cleaning?			
Hair should be brushed regularly to remove dirt and dead hair.	8 (8.5)	17 (18.1)	
Oily hair should be washed more often.	25 (26.6)	32 (34.0)	**p=0.046**
Hair should be brushed quickly and strongly while drying.	32 (34.0)	28 (29.8)	
It should be washed twice a week so that a normal hair oil balance is not disturbed.	29 (30.9)	17 (18.1)	

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Which is the right practice for eye cleaning?			
Only eyelash bottoms of the eyes should be cleaned with water and soap during each bath.	6 (6.4)	10 (10.6)	
In each bath, eyes should be cleaned by rubbing with soap and water.	37 (39.4)	38 (40.4)	p=0.094
The eyes do not need special care, if necessary, only the eye secretion accumulating in the eyelash bottoms should be removed.	22 (23.4)	22 (23.4)	
Only eyes should be cleaned with soap and water in the morning.	29 (30.9)	24 (25.5)	
Which is the right practice for nose cleaning in a healthy individual?			
It should be cleaned by inhaling normal saline into the nostrils.	20 (21.7)	13 (14.1)	
Nose wastes should be removed by blowing with running water or a tissue.	63 (68.5)	66 (71.7)	p=0.113
Only after bathing, nose wastes should be cleaned with a tissue.	6 (6.5)	10 (10.9)	
The nostrils should be cleaned with the help of a foreign object	3 (3.3)	3 (3.3)	
Which is the appropriate method of hand washing according to the cleaning rules?			
It is sufficient to wash your hands by rubbing between the fingers with water.	1 (1.1)	1 (1.1)	
Hands should be washed with an alcoholic solution starting from the wrist level and rubbing between the fingers.	11 (11.7)	11 (11.7)	p=0.401
Hands should be washed with water only for 15-20 seconds before and after each work.	30 (31.9)	24 (25.5)	
Hands should be washed starting from the wrist level with warm water and soap, rubbing between the fingers.	52 (55.3)	58 (61.7)	
Which one is the right practice for cutting the fingernails and toenails?			
Both should be cut straight.	44 (46.8)	44 (46.8)	
Both should be cut rounded.	17 (18.1)	23 (24.5)	p=0.270
Fingernails should be cut rounded and toenails straight.	24 (25.5)	19 (20.2)	
Fingernails should be cut straight and toenails should be cut rounded.	9 (9.6)	8 (8.5)	
Which explains the importance of work clothes in terms of cleanliness and hygiene the best?			
Work uniforms protect other clothes from wear and tear.	5 (5.3)	12 (12.8)	
Work uniforms prevent harmful microorganisms from entering the body.	10 (10.6)	10 (10.6)	p=0.175
They keep other clothes clean and reduce the transmission of harmful microorganisms from the environment to our body and from our body to the environment.	53 (56.4)	55 (58.5)	
Work uniforms make the employee look clean and neat.	26 (27.7)	17 (18.1)	
Would you like to get information about personal hygiene?			
Yes	76 (80.9)	78 (83.0)	p=0.754
No	18 (19.1)	16 (17.0)	
What kind of soap do you usually prefer to wash your hands?			
Soap bar	37 (39.4)	30 (31.9)	p=0.143
Liquid soap	57 (60.6)	64 (68.1)	
How often do you change your socks?			
Every day	87 (92.6)	88 (93.6)	
Once a week	4 (4.3)	3 (3.2)	p=0.905
When it gets dirty and stained	3 (3,2)	3 (3,2)	
Face Towel			
My own	78 (83.0)	88 (93.6)	**p=0.002**
Commonly used at home	16 (17.0)	6 (6.4)	
Foot Towel			
My own	74 (78.7)	91 (96.8)	**p=0.001**
Commonly used at home	20 (21.3)	3 (3.2)	
Bath Towel			
My own	89 (94.7)	90 (95.7)	p=1.000
Commonly used at home	5 (5.3)	4 (4.3)	
Toothbrush			
My own	87 (92.6)	93 (98.9)	p=0.070
Commonly used at home	7 (7.4)	1 (1.1)	
Hand soap (liquid)			
My own	35 (37.2)	45 (47.9)	
Commonly used at home	54 (57.4)	46 (48.9)	**p=0.041**
Commonly used in the workplace	5 (5.3)	3 (3.2)	
Bath washcloth			
My own	83 (88.3)	86 (91.5)	p=0.453
Commonly used at home	11 (11.7)	8 (8.5)	

** McNemear Test was performed in binary comparisons and McNemear-Bowker Test was employed in multiple comparisons.** Percentage of column is taken. BT: Before training, AT: After training*

Table III shows the distribution of changes in hygiene behaviors according to the age of the participants.

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Table III. *Distribution of changes in hygiene behaviors according to the age of the participants (N = 94)*

Hygiene Behavior	n	Age *				Test Value
		25↓	26 – 35	36 – 45	46 ↑	
		n (%)	n (%)	n (%)	n (%)	
How often do you visit the dentist?						
BT						
Never	20	2 (40.0)	2 (7.7)	8 (17.0)	8 (50.0)	
In three months or less	17	2 (40.0)	7 (26.9)	8 (17.0)	0 (0.0)	$\chi^2 = 18.186$
Once in six months	34	1 (20.0)	9 (34.6)	20 (42.6)	4 (25.0)	**p = 0.033**
In a year or more	23	0 (0.0)	8 (30.8)	11 (23.4)	4 (25.0)	
AT						
Never	16	1 (20.0)	1 (3.8)	10 (21.3)	4 (25.0)	
In three months or less	27	3 (60.0)	9 (34.6)	12 (25.5)	3 (18.8)	$\chi^2 = 11.416$
Once in six months	28	1 (20.0)	11 (42.3)	13 (27.7)	3 (18.8)	p = 0.248
In a year or more	23	0 (0.0)	5 (19.2)	12 (25.5)	6 (37.5)	

. Percentage of column is taken. BT: Before – training, AT: After-training*

4. DISCUSSION

In this study, it was aimed to determine the personal hygiene habits of the cleaning staff working in a university and to evaluate the effectiveness of the training conducted. The population of the research consisted of 97 cleaning staff working in a state university in a provincial center, and the sample of the study consisted of cleaning staff (n=94) working in the university during the data collection process who responded to the questionnaire before and after the training. The participation rate in the research was 96.9%.

The participants (50.0%) in the research were between 36-45 years old, and 27.7% of the participants were females. In terms of before-training and after-training, it was determined that the variables other than the dependent variables of the frequency of going to the dentist, the knowledge on wrong practice related to hair cleaning, personal or common use of the face towel, foot towel and hand soap did not display any difference.

If we are to examine the contributions provided in terms of hygiene after the training one by one, it was determined that there was an increase of approximately 10% after the training in the rate of those who said "It is necessary to visit the dentist in 3 months or less". On the other hand, the training did not have an impact on the participants in terms of the frequency of tooth brushing and changing tooth brush. In an interventional study, Coşkun and Kara reported that 70% of students brushed their teeth twice a day and more, that 59.5% changed tooth brushes every 1-3 months, and that these rates increased significantly after training [5]. Muttappillymyalil et al., stated that 84.6% of adolescents brushed their teeth twice a day in their research titled 'Oral Health Behavior Among Adolescents in Kerala / India' [6]. In the study, they also reported that 45.5% of the adolescents stated that they visited the dentist regularly.

The purpose of hand hygiene, which is accepted in personal hygiene practices, is to ensure the disinfection of chemical and physical pests and microorganisms that cause infections. When one cleans his/her hands with water only to have hand hygiene, she/he tries to remove those pathogens through mechanical effect, but complete cleaning cannot be ensured. Therefore, it is mandatory to use soap with water in personal cleaning. Personal hygiene practices and preventive health services are known to reduce certain infections. According to WHO, basic hygiene behaviors such as washing hands with soap, removing stools safely and using clean water are beneficial for improving health [7]. Soap is one of the most effective methods not only for disinfection of the hands but also for the removal of harmful contaminants with allergic effects (nickel, iron and other allergen metals and powders) [8]. Although, normal solid hand soaps and liquid soaps are not different in terms of their effects, soap bars can be sources of contamination due to the environment where they are kept and people leaving the soap uncleaned after using it. Therefore, especially in public places, liquid soaps should be preferred for personal hygiene [9]. In our study, although, the difference was not significant, the rate of cleaning staff choosing liquid soap during hand washing increased by 8% after the training. The rate of those who stated that hand soap should belong to the person was found to increase by 10% in the after-training group, and this difference was significant in terms of comparison between before-training and after-training periods.

In our study, it was determined that the number of participants in the after-training group who stated that the face towel should belong to the person, increased by 10.6%. On the other hand, it was also found that the after-training group stated that the foot towel should belong to the person with an increase of approximately 18.1%. In the study conducted in 2017 on personal hygiene habits of elementary school students in our country, the rate of using towels in the urban area was found to be 63.3% and 36.7% in the rural area [10]. In their research in which they investigated the students of two different primary schools in Istanbul, Önsüz and Hıdıroğlu, found that 48.3% of the students studying in Ümraniye had personal towels. In contrast, they determined that 59.4% of the students studying in Üsküdar had a personal towel [4]. In their interventional research, Coşkun and Kara found that the rate of students' using

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towels after washing their hands was 93.9% before training, while this rate increased to 95.0% after training [5].

The appearance of the hair usually gives an idea about the general health of the people and their level of personal hygiene. People with messy and dirty hair are often inadequate in terms of hygiene practices. In such cases, infection-causing factors and parasites can easily be transmitted to dirty hair and scalp. Hair should normally be washed at least once or twice a week. Oily hair types should be washed frequently and appropriate hair washing products should be used [11]. Regarding hair cleaning, it was determined that the participants in the after-training group said that "The hair should be brushed regularly in order to remove dirt and dead hair" with an increase of more than 10%. The rate of increase in those who said that oily hair should be washed more frequently was around 8% in the training group. In different studies conducted on the frequency of taking a bath among primary school students, the frequency of taking a bath every three days and above was found to be between 57.4% and 69.5% [10,12]. It was found that 67.8% of the students participating in this research took a bath in three days or more before the training, and this rate went up to 77.8% after the training. In Arat et al's. research titled 'Personal Hygiene Practices of Boarding Elementary School Second Level Students', it was found that 38.2% of students washed their hair once every two days and 29.9% every three days [13]. Coşkun and Kara also found in their research that 67.8% of the students who participated in the research had a bath every three days or more before the training, and this rate increased to 77.8% after the training [15]. Our findings are consistent with literature experiments [14-16].

Conclusion and Suggestions

In the research, the effect of health training provided to cleaning staff on knowledge and behavior was examined. As a result of the research, it was observed that there was a general positive increase related to the hygiene issues in the level of knowledge and behavior of the cleaning staff.

In conclusion, there is a lack of training for the cleaning staff included in this research regarding the work they do and personal hygiene. The rate of using personal materials related to the working environment and using personal materials in the home environment is low. It seems that some behaviors regarding cleaning / hygiene / health are inadequate. Measures and training programs should be increased for the personnel working in cleaning jobs to perform in a more effective and healthy manner. In addition, they should be provided with opportunities to develop hygiene and gain positive behavior starting from childhood. Periodic training sessions should be held within the scope of occupational health for those in this age group.

Compliance with Ethical Standards

Ethical Approval: This study was approved by Bingöl University Ethics Committee (Approval date and number: 15.04.2019; 92342550/044 – E.8055).

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Conflict of Interest: The authors have no potential conflicts to disclose.

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Case Report

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A rare cutaneous lesion in the neonatal period: The non-Langerhans cell histiocytosis

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ABSTRACT

The non-Langerhans cell histiocytosis (non-LCH) is a group of diseases characterized by cutaneous involvement in the neonatal period. The non-LCH affects less than 1 in 200,000 children born each year. A definitive diagnosis is important for the treatment of the disease. Therefore, skin biopsies should be performed in neonates with cutaneous lesions as early as possible. Only in the presence of cutaneous involvement without systemic involvement, it does not require any treatment, because the lesions can mostly self-heal. Here, we present a neonate diagnosed with non-LCH following a skin biopsy.

Keywords: Histiocytic disorders, Neonatal, non-Langerhans cell histiocytosis

1. INTRODUCTION

The histiocytic syndromes consist of a group of disorders that share in common the proliferation of cells of the monocyte/macrophage lineage. It has been conventional to divide the histiocytoses into two separate groups: Langerhans cell histiocytosis (LCH) and non-LCH. Histiocytosis affects 1 in 200,000 children born each year; non-LCH is much more rare [1]. The non-LCH are a group of disorders defined by the accumulation of histiocytes that do not meet the phenotypic criteria for the diagnosis of Langerhans cells.

2. CASE REPORT

A 2-day-old female neonate, born at 39 weeks of gestation with normal spontaneous vaginal delivery was referred to our hospital due to skin lesions. She was born with pinkish – red, palpable lesions with 5-15 mm in diameter, scattered all over the body including scalp (Figures 1, 2). She was being breastfed by her mother. On physical examination, her vital signs were normal except palpable skin lesions. Laboratory tests, including complete blood count, peripheral smear, urinalysis, liver and kidney function tests, coagulation tests, procalcitonin, viral serology were all normal. Toxoplasmosis, other (including syphilis), rubella,cytomegalovirus, herpes simplex virus (TORCH) and total immunoglobulin (IgM) tests were all negative. Transabdominal and transcranial ultrasonographies were normal. Skin biopsy was performed and CD68 and S100 markers were found to be positive. As a result, a diagnosis of non-LCH was made. Birbeck granules were absent in the cells (Figures 3, 4). In the follow-up of the patient, the lesions disappeared at the age of 6 months. At 10 months of age she was with no evidence of relapse. She is being followed-up at the Neonatology and Dermatology outpatient clinics. Written informed consent was obtained from the parents for publication of this case report including the photographs.

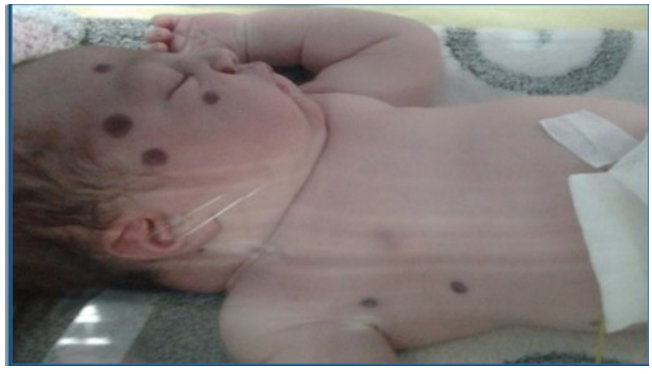

***Figure 1**. Palpable skin lesions*

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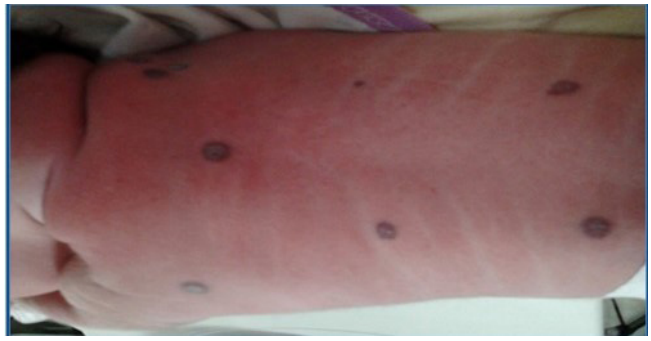

Figure 2. *Palpable skin lesions*

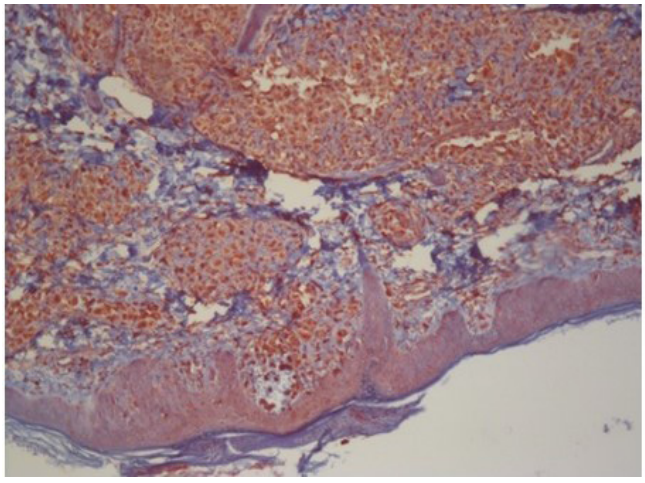

Figure 3. *CD68 staining confirmed the existence of histiocytes*

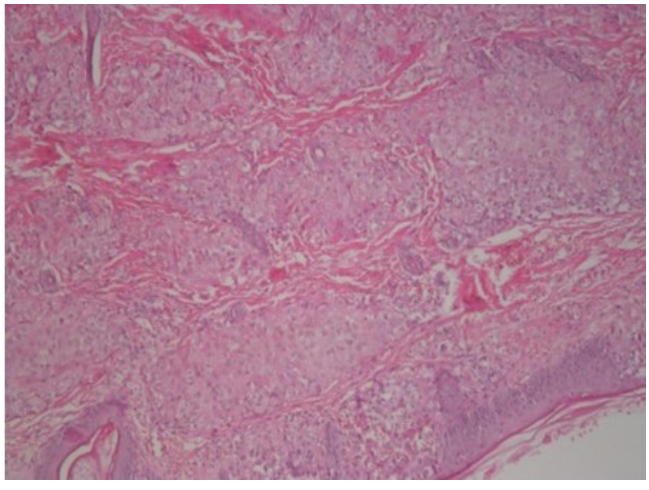

Figure 4. *Sections of the lesion with histiocytosis (H and E x 100)*

3. DISCUSSION

The non-LCH is a group of diseases with cutaneous involvement in the neonatal period [1,2]. Dermal dendrocyte infiltration is seen in this group of diseases, but typical Langerhans cells are absent. As reported by Classen, et al., some subtypes of these disorders typically occur early in life and tend to resolve spontaneously [3]. Our patient, also had multiple palpable lesions all over her body, with no systemic involvement. The lesions spontaneously disappeared by 6 month of age.

Clinically, the non-LCH can be divided into 3 groups. The first group that predominantly affects the skin; the second group that affects the skin but has a major systemic component such as hepatic, skeletal, central nervous system or lung involvement; and the third group that primarily involves extra-cutaneous sites, although skin may be also involved [4]. Some subtypes may be disseminated and life-threatening [5]. In childhood, only cutaneous involvement form limits itself [6,7]. In our patient, non-LCH affected skin predominantly.

Prompt evaluation of disease extent upon diagnosis is mandatory for risk-adapted treatment. Although, the prognosis cannot be predicted after the diagnosis, the course of the disease varies from spontaneous recovery to chronic course or fulminant deterioration [8].

Lesions which occur in the first 6 months of life can be either solitary or multiple. Lesions are about 5-20 mm in diameter, pinkish-red in color, firm, solid and limited well. In the follow-up, lesions can fade or they can heal with atrophic scars or hyperpigmentation in months or years. Diagnosis is made histopathologically [3].

The etiology and pathogenesis of non-LCH and its various clinical forms remain obscure. Viral, immunologic, and neoplastic mechanisms have been proposed, but none of them have been conclusively proven. Lesions originate from histiocytes and monocyte/macrophage derived giant cells. Macrophage surface markers of these patients are positive for CD68 and HAM [9,10]. CD68 and S100 markers were positive in the specimen of our patient. Extracutaneous involvement is rare; eye, spleen, testicles, penis, pericardium, gastrointestinal system and kidneys may be involved. A patient with non-LCH should be evaluated for extracutaneous involvement. Radiological, biochemical and hematological investigations must be done to exclude xanthoma disseminatum, generalized eruptive histiocytoma and pancytopenia [2]. Our patient did not have any extracutaneous lesions and pancytopenia. No pathological findings were detected in her cranial and abdominal ultrasonograpy scans. Treatment for extracutaneous involvement may be required to decrease the mass effect of the lesions. Advanced treatment regimens and surgical approach may be considered in non-LCH, when there is major systemic involvement along with cutaneous involvement [11]. Only in the presence of cutaneous involvement, it does not require any treatment, as the lesions resolve spontaneously. The lesions in our patient disappeared without any treatment.

The non-LCH can mimic a number of neonatal skin lesions; including neonatal pustular melanosis, perinatal herpes simplex and Listeria monocytogenes, congenital candidiasis, neonatal varicella, syphilis, erythema toxicum, incontinentia pigmenti, neonatal disseminated hemangiomatosis, erythropoiesis dermica, and congenital leukemia [12]. Gram stain, potassium hydroxide

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(KOH) and Tzanck preparations, and bacterial, viral, and fungal cultures help to differentiate the non-LCH from other disorders. TORCH serologies help to rule out congenital intrauterine infections. A skin biopsy specimen is, however, necessary for confirmation of the diagnosis.

The differential diagnosis of the disseminated histiocytoses is sometimes difficult because they occur with overlapping clinical and pathological findings. Nevertheless, the histiocytoses must be clearly differentiated from one another because they require different forms of treatment approaches and have different outcomes [9,10]. Although, the disease shows spontaneous involution, careful evaluation for systemic disease and long-term follow-up to detect relapse are essential in the management of these patients.

We would like to emphasize that non-LCH which predominantly affects the skin is benign and disappears without any treatment. A skin biopsy specimen is, however, necessary for confirmation of the diagnosis.

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