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The effects of a persimmon leaf oral supplement (Persimonal®) on cardiovascular health: a randomized, double-blinded, placebo-controlled study

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Abstract

Introduction: The present randomized, double-blinded, placebo-controlled study investigated the effects of Persimonal®, a persimmon leaf oral supplement on cardiovascular health.

Methods and materials: Eighty-six (86) subjects, male and female aged 40-75 years of age inclusive, with borderline hypertension and/or borderline high total cholesterol or high LDL, were enrolled onto the study and seventy-three (73) subjects completed the study. Qualified subjects returned to the testing facility for baseline (Day 0). Following review of qualification, the test product, diary, and instructions for use were dispensed according to the randomization for the study. Subjects returned for visits on Days 7, 30, 60, 90, 120, 150, and 180. Subjects had the same procedures completed depending on which group they were included in (blood pressure, blood draw or both). On Day 180, all subjects had a blood pressure check and blood drawn for a lipid profile for safety.

Results: Systolic and diastolic BP analysis indicated subjects assigned treatment with Persimonal® had statistically significant reductions in mean blood pressure values compared to baseline values at all study assessment timepoints for systolic BP and diastolic BP, except for day 07 in this last parameter. Between treatment analysis indicated statistically significant differences in systolic and diastolic blood pressure values favoring Persimonal®. The lipid profiles of the subjects randomized to treatment with Persimonal® showed a statistically significant reduction in the mean LDL and in the mean total cholesterol values compared to mean baseline values at Days 07, 60, 90 and 120 for LDL and Days 07, 30 and 120 for total cholesterol.

Conclusion: The results of the study provide data supporting the view that Persimonal® may be administered to patients as a potential cardiovascular dietary supplement. Further research will elucidate additional benefits from this multifunctional source.

Key Words: *Persimmon Leaf Extract, Persimonal, Cardiovascular health, Blood pressure, Cholesterol.*

Introduction

Persimonal® is a new multifunctional advanced botanical extract of highly therapeutic bioactive compounds of flavonoids, polyphenols, tannins, terpenoids, gallic acid, and carotenoids, which are naturally sourced from leaves of *Diospyros kaki* Thumb. (Persimmon leaves).

These polyphenolic compounds are known to be anti-oxidative, anti-inflammatory, anti-carcinogenic, anti-mutagenic, and cardioprotective, boosting the immune system and nourishing the cells with a daily intake of this highly nutritious supplement to maintain a healthy cardiovascular system (HAN *et al*., 2002).

Persimmon (*Diospyros kaki* Thumb.) is a traditional medicinal plant, widely cultivated in China, Japan, and South Korea and has been commonly used in folk medicine in East Asia, owing to its different pharmacological activities; mainly antihypertensive (KAWAKAMI *et al*., 2010), antioxidant (SUN *et al*., 2011), and vasorelaxant effects (KAWAKAMI *et al*., 2011).

Persimmon fruits possess high levels of dietary fiber, vitamin C, catechin and gallocatechin whereas Persimmon Leaf Extract mainly consists of caffeine, chlorophyll, flavonoids (including

therapeutic constituents, astragalin, kaempferol, quercetin), organic acids, phenolic compounds, tannins, and vitamins (LEE *et al*., 2006). As a folk medicine, Persimmon Leaf Extract and whole persimmon leaf have widely used to treat hypertensive disease and apoplexy syndrome in Asia (BAE *et al*., 2015).

Although, from the research perspective, there is convincing evidence that Persimmon Leaf Extract ingestion may improve cardiovascular conditions, and, based on the findings that Persimmon biomarkers are absorbed in their molecular form, accumulating in different tissues, it might be reasonable to investigate a new multifunctional Persimmon Leaf Extract as a nutritional supplement. Thus, the aim of this single-center investigation is to extend these earlier findings with Persimonal®.

Methods and materials

Study design

This was a randomized, double-blind, placebo-controlled study that utilized blood pressure monitoring and/or blood cholesterol assessments to demonstrate the potential of a nutritional supplement to aid in cardiovascular health improvement when used daily for approximately 180 days. The subjects as well as the Principal Investigator were blinded to whether the subject received active test article or placebo.

Inclusion criteria
Male or female, aged 40-75 years of age
Subject has borderline hypertension and/or borderline total cholesterol, or borderline LDL levels as determined by the consulting physician. If values are over the maximum for the study, staff are asked to refer the subject to their PCP
Subject can either be newly diagnosed and on no medication for the condition or on medication for a previous diagnosis (stable on the same medication for the last 3 months) and still have borderline hypertension, total cholesterol, or LDL levels
Subject has provided written informed consent
Subject has come to Visit 1 fasting for 9-12 hours
Subject can comply with study product requirements and attending all visits
Female subjects of child-bearing potential agree to use an adequate method of birth control which would include, 1) Systemic birth control (subjects must have been taking the same type of birth control for at least 3 months prior to entering the study and must not change type of birth control during the study); 2) Condom with spermicide; 3) IUD; 4) Abstinence. Females not of child-bearing potential (hysterectomy, bilateral tubal ligation, or no menses for more than 12 months) do not need to agree to use birth control

Exclusion criteria
Female subject who is pregnant, nursing, or planning to become pregnant (by verbal response only)
Females who have given birth within the past year
Subject has participated in another supplement study in the past 30 days
Subject is under treatment for known health issues which may include, but not be limited to: Asthma, Diabetes, Insomnia, Autism, Vertigo, Thyroid Issues, Migraines, Bi- Polar Disorder or episodes of Mania, Depression, Cancer or history of Cancer, Heart Disease or undergoing any form of hormone therapy
History of malignant disease
Known sensitivity/allergy to the test article, similar materials, or their constituents
Current use of recreational drugs
Principal Investigator deems the subject an unsuitable candidate for this study

Prohibitions and restrictions
Subject agrees to not start any new medications or herbal supplements without discussing first with study staff
Subject agrees to remain on a stable diet (i.e., no dietary changes from the last 30 days)
Subject who is in the high cholesterol/high LDL group agrees to come to every visit fasting for at least 9 hours prior to their visit for blood draw. Subjects may drink plenty of water to stay hydrated but must refrain from eating or drinking anything other than water prior to every visit

Selection of subjects

Subjects had to satisfy the following inclusion and none of the exclusion criteria and had to accept the prohibitions and restrictions to be enrolled onto the study. The suitability of each subject to participate was confirmed prior to their acceptance onto the study by completion and review of a study specific pre-study questionnaire along with the results of the baseline blood pressure assessment and baseline Cholesterol blood work results.

Subject withdrawal

The participation of a subject in this study was discontinued for any of the following reasons:

- the subject wished to withdraw from study participation.
- if, in the opinion of the Investigator, it was in the best interest of the subject.
- suspected adverse effects from the test article.
- inter-current illness.
- violation of the prohibitions and restrictions.
- development of an exclusion criteria.

Subjects were free to withdraw at any time and needed not give a reason, however, every reasonable attempt was made to ascertain such reasons. The data for those subjects who were withdrawn was included in the final clinical data but was excluded from final data analysis. Subjects were not followed up with after their withdrawal from the study, except in the case of a serious adverse event. Withdrawn subjects were not replaced.

One hundred and thirty-eight (138) male and female subjects were consented and screened for study eligibility. Eighty-six (86) qualified subjects were enrolled on to the study and assigned test product. Seventy-three (73) subjects completed the study. The following tables are a summary of the demographics for subjects who were enrolled into each of the treatment groups for the study.

Table 1. Demographic summary

Borderline Hypertension Group – 1			
	All subjects	Active	Placebo
Age			
N	29	16	13
Mean	55.6	52.6	59.2
Standard Deviation	10.2	8.4	11.2
Median	55.0	50.0	60.0
Range	40.0 to 74.0	40.0 to 69.0	40.0 to 74.0
Race – N (%)			
White	22 (75.9)	13 (81.3)	9 (69.2)
Black	5 (17.2)	2 (12.5)	3 (23.1)
Bi-racial	2 (6.9)	1 (6.3)	1 (7.7)
Asian	0 (0.0)	0 (0.0)	0 (0.0)
Gender – N (%)			
Female	21 (72.4)	13 (81.3)	8 (61.5)
Male	8 (27.6)	3 (18.8)	5 (38.5)

High Cholesterol Group – 2			
	All subjects	Active	Placebo
Age			
N	4	2	2
Mean	53.5	57.0	50.0
Standard Deviation	5.7	4.2	5.7
Median	54.0	57.0	50.0
Range	46.0 to 60.0	54.0 to 60.0	46.0 to 54.0
Race – N (%)			
White	4 (100)	2 (100)	2 (100)
Black	0 (0.0)	0 (0.0)	0 (0.0)
Bi-racial	0 (0.0)	0 (0.0)	0 (0.0)
Asian	0 (0.0)	0 (0.0)	0 (0.0)
Gender – N (%)			
Female	4 (100)	2 (100)	2 (100)
Male	0 (0.0)	0 (0.0)	0 (0.0)
Borderline Hypertension and High Cholesterol Group – 3			
	All subjects	Active	Placebo
Age			
N	53	37	16
Mean	52.6	52.2	53.6
Standard Deviation	9.1	9.2	9.1
Median	51.0	51.0	50.0
Range	38.0 to 73.0	38.0 to 73.0	40.0 to 69.0
Race – N (%)			
White	51 (96.2)	35 (94.6)	16 (100)
Black	1 (1.9)	1 (2.7)	0 (0.0)
Bi-racial	0 (0.0)	0 (0.0)	0 (0.0)
Asian	1 (1.9)	1 (2.7)	0 (0.0)
Gender – N (%)			
Female	42 (79.2)	30 (81.1)	12 (75.0)
Male	11 (20.8)	7 (18.9)	4 (25.0)

Test article

The test articles utilized in this study were Persimonal® Persimmon Leaf Extract (Lot# PLE-191201) and Placebo (Lot# PLE-191202). Both were provided in identical plain packaging and marked with the lot number only (located on the underside of each container dispensed to subjects) that identified treatment assigned so that both the subjects and the PI were blinded to treatment.

Subjects were instructed to take 2 capsules daily, containing 150mg each, with water or juice, preferably at the same time of day each day. The subjects were asked to take the test product with a meal and asked to return to each visit with their study product and diary for compliance assessments. Subjects assigned to the high Cholesterol or high LDL group were also instructed to arrive at each subsequent visit fasting for at least 9 hours prior to arrive at the testing center.

Study procedures

Visit 1 – Screening

Subjects attended the test center for screening and completed the informed consent form (ICF). Subjects were asked to provide their medical history and any concomitant medications being taken. They were screened for eligibility (inclusion/exclusion criteria) to be on the study, sitting blood pressure was taken, and a blood draw for lipid profile assessment was collected. Following assessments, subjects were dismissed. The blood pressures captured, and the blood lipid panel results collected at visit 1 were the baseline values used for the study.

The consulting physician reviewed the screening information collected (including blood pressure and blood lipid profile panel results) and indicated which subjects qualified for study enrollment. Subjects who qualified for both the hypertension and cholesterol group comprised the third group. Subjects were contacted by the testing facility to notify them if they qualified for study enrollment. Subjects who qualified were given an appointment time and instructions for their next visit.

Subjects who did not qualify due to blood pressure results that were too high or lipid profile results that were in excess of the consulting physician's recommendation were notified by study staff and were encouraged to discuss their results with their Primary Care Physician (PCP). Subjects who were disqualified were allowed to request a copy of their screening results so that they could share the results with their PCP.

Visit 2 – Enrollment

Qualifying subjects returned to the test center at their scheduled time. Subjects were queried for any changes to their health or medication and eligibility with inclusion/exclusion criteria was confirmed. Subjects were then assigned into one of three groups – those with borderline hypertension; those with high total cholesterol or LDL levels, or subjects who had both borderline hypertension and high cholesterol/high LDL. Subjects were issued the test product (Persimonal® Persimmon Leaf Extract or Placebo) in numerical bottle number order as they qualified for study enrollment as well as issued with written instructions on when and how to take the test product, and a diary to document use was also provided. Subjects were given a return appointment for their next visit and instructions to arrive at that visit fasting if they were in the groups requiring blood draw.

Visits 3 to 8 - (Days 7 (± 2 days), 30, 60, 90, 120, and 150 (± 5 days)

Subjects returned to the test center with their assigned test product and diary and were queried for any changes in their health or medications. Subject's product and diary were collected and reviewed for compliance. If necessary, subjects were issued new test product per their treatment assignment and were given a new diary to complete as appropriate. Subjects underwent either a blood pressure check or blood draw or both depending on what group they were assigned and were given an appointment time for their next visit. If subjects were found to be non-compliant with test article use, they were counselled on the proper use of the test supplements. If continued compliance was an issue (barring any side effect experienced) the subject was discontinued from the study.

Visit 9 - (Day 180 (± 5 days)

Final Visit: Subjects returned to the test center with their product and diary and were queried for any changes in their health or medications. Subject's test product and diary were collected and

reviewed for compliance. Subjects underwent either a final a blood pressure check and/or a blood draw. After all assessments were completed, the subject's participation in the study was considered complete.

Blood Pressure Assessments

Subjects were asked to sit in a chair and rest for five minutes prior to any blood pressure measurement being taken. The arm that the blood pressure measurement was taken at visit 1 was documented in the source. At each subsequent visit, blood pressure was taken from the same arm and in the same way as the first visit. Blood pressure was taken with an automatic blood pressure monitoring device. Once the blood pressure was confirmed for each subject at screening, the medical consultant used the following guidance for approval of a subject as having borderline hypertension and was allowed to continue in the study: 120/80 < Subject's BP ≤ 140/90.

If the systolic and diastolic blood pressure readings at any time met the following, the subjects blood pressure should have been re-taken. If the readings recorded were confirmed, the medical consultant was contacted, and an assessment of the subject's safety was made. If appropriate, the subject was stopped from further study participation. The subjects were referred to their Primary Care Provider for follow-up: Systolic: Less than or equal to 90 mmHg/Diastolic less than or equal to 60 mmHg; or Systolic: Greater than 140 mmHg/ Diastolic greater than 90 mmHg.

If BP was 180/110 (for either systolic or diastolic) or higher, the subject may have been experiencing a hypertensive crisis. If this was the case, the subject was referred for immediate medical attention.

Laboratory Assessments (Blood Draws)

Subjects were asked to fast for 9-12 hours prior to every blood draw (no food or drinks except for water). It was important to remind subjects to still drink plenty of water during the fasting period to prevent dehydration. If the subject came to a visit without fasting the required 9-12 hours, the visit was to have been rescheduled within the visit window of that specific visit.

A standard lipid profile (including total cholesterol, triglycerides, HDL, LDL, cholesterol/ HDL ratio calculated, and non-HDL Cholesterol calculated) was completed at each required visit. The medical consultant reviewed the lipid panel results for each subject at screening and used the following guidance for approval of a subject as having high cholesterol or high LDL based on the lipid panel results as follows: Total Cholesterol between 5 mmol/L and 6 mmol/L; LDL between 3 mmol/L and 4 mmol/L.

For all subsequent lipid panel results, any levels observed above the initial baseline results at the completion of the study were reviewed by the medical consultant and if warranted, the subject was referred to their Primary Care Physician for follow-up.

Method of statistical analysis

The source data are the LDL, Total Cholesterol and Systolic and Diastolic blood pressure readings. There are 3 treatment groups, within each treatment group subjects were assigned to either Treatment (Persimonal® Persimmon Leaf Extract Lot# PLE-191201) or Placebo (Lot #PLE-191202).

Separate analyses were conducted separately for the following three groups:

Borderline Hypertension Group – 1	Systolic and Diastolic blood pressure readings were taken at Baseline and post-treatment at Days 7, 30, 60, 90, 120, 150 and 180. This group did not have LDL or Total Cholesterol assessments.
High Cholesterol Group – 2	LDL and Total Cholesterol Assessments were taken at Baseline and post-treatment at Days 7, 30, 60, 90, 120, 150 and 180. Blood pressure readings were done at Baseline and 180 days as a Safety check only, data was not entered nor listed.
Borderline Hypertension and High Cholesterol Group – 3	LDL and Total Cholesterol Assessments and Systolic and Diastolic blood pressure readings were taken at Baseline and post-treatment at Days 7, 30, 60, 90, 120, 150 and 180.

The post-treatment changes from baseline were analyzed within-treatment using Student's t-test for paired data and between-treatment using analysis of covariance with the baseline measurement as the covariate.

All statistical tests of hypothesis employed a level of significance of 0.05 and no adjustments were made for the number of tests performed. The number of subjects that were enrolled in group 2 was not sufficient to perform statistical analysis within the treated vs placebo group.

Table 2. Systolic Blood Pressure: Significant within-treatment t-test p-values are noted with an asterisk (*). Significant between-treatment ANCOVA p-values are noted with two asterisks (**).

Treatment	Evaluation	N	Treatment Mean	Mean Difference from Baseline	Within-Treatment t-test p-value	Percent Change Mean [1]	Percent Change Subject [2]	Number Improved	Percent Improved	Difference Between Treatments	Between-Treatment ANCOVA p-value
Persimonal®	Baseline	16	149.63								
	Day 7	16	142.44	-7.19	0.0016*	-4.8%	-4.7%	14	87.5%	-3.88	0.3220
	Day 30	16	126.38	-13.25	<0.0001*	-8.9%	-8.8%	15	93.8%	-3.40	>0.5000
	Day 60	16	134.63	-15.00	0.0012*	-10.0%	-9.6%	14	87.5%	-7.62	>0.5000
	Day 90	16	129.94	-19.69	<0.0001*	-13.2%	-12.9%	15	93.8%	-13.23	0.0095**
	Day 120	16	131.75	-17.88	<0.0001*	-11.9%	-11.5%	14	87.5%	-10.95	0.0556
	Day 150	16	129.44	-20.19	<0.0001*	-13.5%	-13.1%	15	93.8%	-14.34	0.0326**
	Day 180	16	130.13	-19.50	<0.0001*	-13.0%	-12.7%	15	93.8%	-16.73	0.0043**
Placebo	Baseline	13	141.38								
	Day 7	13	138.08	-3.31	0.0246*	-2.3%	-2.3%	11	84.6%		
	Day 30	13	131.54	-9.85	0.0009*	-7.0%	-6.8%	13	100.0%		
	Day 60	13	134.00	-7.38	0.0105*	-5.2%	-5.0%	11	84.6%		
	Day 90	13	134.92	-6.46	0.0211*	-4.6%	-4.2%	11	84.6%		
	Day 120	13	134.46	-6.92	0.0162*	-4.9%	-4.6%	11	84.6%		
	Day 150	13	135.54	-5.85	0.1654	-4.1%	-3.8%	8	61.5%		
	Day 180	13	138.62	-2.77	0.4139	-2.0%	-1.7%	8	61.5%		

** Significant change from baseline*
*** Significant change between treatments*

Table 3. Diastolic Blood Pressure: Significant within-treatment t-test p-values are noted with an asterisk (*). Significant between-treatment ANCOVA p-values are noted with two asterisks (**).

Treatment	Evaluation	N	Treatment Mean	Mean Difference from Baseline	Within-Treatment t-test p-value	Percent Change Mean [1]	Percent Change Subject [2]	Number Improved	Percent Improved	Difference Between Treatments	Between-Treatment ANCOVA p-value
Persimonal®	Baseline	16	93.81								
	Day 7	16	91.88	-1.94	0.1806	-2.1%	-1.9%	12	75.0%	0.37	0.1873
	Day 30	16	86.38	-7.44	<0.0001*	-7.9%	-7.8%	14	87.5%	-0.28	0.4118
	Day 60	16	85.81	-8.00	0.0032*	-8.5%	-8.1%	14	87.5%	-3.69	>0.5000
	Day 90	16	86.44	-7.38	0.0002*	-7.9%	-7.7%	14	87.5%	-4.30	0.2571
	Day 120	16	85.38	-8.44	<0.0001*	-9.0%	-8.7%	15	93.8%	-3.98	>0.5000
	Day 150	16	85.31	-8.50	0.0011*	-9.1%	-8.5%	14	87.5%	-5.50	>0.5000
	Day 180	16	86.94	-6.88	0.0002*	-7.3%	-7.0%	15	93.8%	-5.80	0.3169
Placebo	Baseline	13	88.54								
	Day 7	13	86.23	-2.31	0.3359	-2.6%	-2.0%	11	84.6%		
	Day 30	13	81.38	-7.15	0.0088*	-8.1%	-7.4%	12	92.3%		
	Day 60	13	84.23	-4.31	0.0882	-4.9%	-4.4%	10	76.9%		
	Day 90	13	85.46	-3.08	0.1174	-3.5%	-3.3%	8	61.5%		
	Day 120	13	84.08	-4.46	0.1300	-5.0%	-4.6%	10	76.9%		
	Day 150	13	85.54	-3.00	0.2512	-3.4%	-2.9%	6	46.2%		
	Day 180	13	87.46	-1.08	>0.5000	-1.2%	-0.6%	5	38.5%		

** Significant change from baseline*
*** Significant change between treatments*

Results and discussion

Borderline Hypertension Group – 1 Systolic

Systolic blood pressure analysis indicated subjects assigned treatment with Persimonal® Persimmon Leaf Extract had statistically significant reductions in mean Systolic blood pressure values compared to baseline values at all study assessment timepoints. Subjects assigned to the Placebo treatment had statistically significant reductions in mean Systolic blood pressure values compared to baseline values at Days 7, 30, 60, 90, and 120. Between treatment analysis indicated statistically significant differences in Systolic blood pressure values favoring Persimonal® Persimmon Leaf Extract at Days 90, 150 and 180.

Borderline Hypertension Group – 1 Diastolic

Diastolic blood pressure analysis indicated subjects assigned treatment with Persimonal® Persimmon Leaf Extract had statistically significant reductions in mean Diastolic blood pressure values compared to baseline values at Days 30, 60, 90, 120, 150, and 180. Subjects assigned to the Placebo treatment had statistically significant reductions in mean Diastolic blood pressure values compared to baseline values at Day 30. Between treatment analysis indicated no statistically significant differences in Diastolic blood pressure values.

High Cholesterol Group – 2 LDL

Due to the low number of subjects enrolled into this treatment group, no analysis was performed on this group.

High Cholesterol Group – 2 Total Cholesterol

Due to the low number of subjects enrolled into this treatment group, no analysis was performed on this group.

Borderline Hypertension and High Cholesterol Group – 3 Systolic

Borderline Hypertension and High Cholesterol Group – 3 Diastolic

Overall, there was no statistically significant changes in mean Systolic or Diastolic blood pressure for subjects assigned to Persimonal® Persimmon Leaf Extract treatment. Subjects assigned to the Placebo treatment had no statistically significant changes in mean Systolic blood pressure values compared to baseline but had statistically significant lower mean Diastolic blood pressure values at day 30 only when compared to baseline. Between treatment comparison showed that the Placebo treatment group had statistically significant lower Diastolic blood pressure values compared to the active treatment at day 30.

Table 4. LDL: Significant within-treatment t-test p-values are noted with an asterisk (*). Significant between-treatment ANCOVA p-values are noted with two asterisks (**).

Treatment	Evaluation	N	Treatment Mean	Mean Difference from Baseline	Within-Treatment t-test p-value	Percent Change Mean [1]	Percent Change Subject [2]	Number Improved	Percent Improved	Difference Between Treatments	Between-Treatment ANCOVA p-value
Persimonal®	Baseline	2	3.55								
	Day 7	2	3.70	0.15	>0.5000	4.2%	3.9%	1	50.0%		0.3784
	Day 30	2	3.70	0.15	0.2048	4.2%	4.2%	0	0.0%		Not done
	Day 60	1	4.50	0.80	Not done	22.5%	21.6%	0	0.0%		Not done
	Day 90	1	1.70	-2.00	Not done	-56.3%	-54.1%	1	100.0%		Not done
	Day 120	1	2.70	-1.00	Not done	-28.2%	-27.0%	1	100.0%		Not done
	Day 150	1	2.50	-1.20	Not done	-33.8%	-32.4%	1	100.0%		Not done
	Day 180	1	2.50	-1.20	Not done	-33.8%	-32.4%	1	100.0%		Not done
Placebo	Baseline	2	3.50								
	Day 7	1	4.05	0.55	0.0577	15.7%	15.7%	0	0.0%		
	Day 30	1	2.80	-0.40	Not done	-11.4%	-12.5%	1	100.0%		
	Day 60	1	2.40	-0.80	Not done	-22.9%	-25.0%	1	100.0%		
	Day 90	1	2.20	-1.00	Not done	-28.6%	-31.3%	1	100.0%		
	Day 120	1	2.70	-0.50	Not done	-14.3%	-15.6%	1	100.0%		
	Day 150	1	3.60	0.40	Not done	11.4%	12.5%	0	0.0%		
	Day 180	1	3.30	0.10	Not done	2.9%	3.1%	0	0.0%		

** Significant change from baseline*

*** Significant change between treatments*

Not done = test not done due to insufficient data (i.e. 1 subject)

Table 5. Total Cholesterol: Significant within-treatment t-test p-values are noted with an asterisk (*). Significant between-treatment ANCOVA p-values are noted with two asterisks (**).

Treatment	Evaluation	N	Treatment Mean	Mean Difference from Baseline	Within-Treatment t-test p-value	Percent Change Mean [1]	Percent Change Subject [2]	Number Improved	Percent Improved	Difference Between Treatments	Between-Treatment ANCOVA p-value
Persimonal®	Baseline	2	5.40								
	Day 7	2	5.70	0.30	Not done	5.6%	5.6%	0	0.0%	-0.05	>0.5000
	Day 30	2	5.45	0.05	0.5000	0.9%	0.9%	0	0.0%	0.35	Not done
	Day 60	1	6.40	0.70	Not done	13.0%	12.3%	0	0.0%		Not done
	Day 90	1	3.30	-2.40	Not done	-44.4%	-42.1%	1	100.0%		Not done
	Day 120	1	4.30	-1.40	Not done	-25.9%	-24.6%	1	100.0%		Not done
	Day 150	1	4.20	-1.50	Not done	-27.8%	-26.3%	1	100.0%		Not done
	Day 180	1	4.20	-1.50	Not done	-27.8%	-26.3%	1	100.0%		Not done
Placebo	Baseline	2	5.80								
	Day 7	1	6.15	0.35	0.2578	6.0%	6.1%	0	0.0%		
	Day 30	1	5.30	-0.30	Not done	-5.2%	-5.4%	1	100.0%		
	Day 60	1	5.10	-0.50	Not done	-8.6%	-8.9%	1	100.0%		
	Day 90	1	5.30	-0.30	Not done	-5.2%	-5.4%	1	100.0%		
	Day 120	1	5.10	-0.30	Not done	-8.6%	-8.9%	1	100.0%		
	Day 150	1	6.30	0.70	Not done	12.1%	12.5%	0	0.0%		
	Day 180	1	5.70	0.10	Not done	1.7%	1.8%	0	0.0%		

** Significant change from baseline*
*** Significant change between treatments*
Not done = test not done due to insufficient data (i.e. 1 subject)

Table 6. Systolic Blood Pressure: Significant within-treatment t-test p-values are noted with an asterisk (*). Significant between-treatment ANCOVA p-values are noted with two asterisks (**).

Treatment	Evaluation	N	Treatment Mean	Mean Difference from Baseline	Within-Treatment t-test p-value	Percent Change Mean [1]	Percent Change Subject [2]	Number Improved	Percent Improved	Difference Between Treatments	Between-Treatment ANCOVA p-value
Persimonal®	Baseline	37	132.22								
	Day 7	37	133.65	1.43	>0.5000	1.1%	2.4%	17	45.9%	3.36	0.2693
	Day 30	35	132.17	-1.06	>0.5000	-0.8%	0.4%	16	45.7%	6.23	0.0360**
	Day 60	35	130.00	-3.23	0.2393	-2.4%	-1.4%	20	57.1%	-7.09	0.2146
	Day 90	34	131.91	-1.12	>0.5000	-0.8%	0.3%	17	50.0%	-5.70	0.3944
	Day 120	35	132.26	-0.97	>0.5000	-0.7%	0.3%	17	48.6%	-10.72	0.0715
	Day 150	33	129.33	-1.94	>0.5000	-1.5%	-0.6%	18	54.5%	-10.34	0.1818
	Day 180	33	127.12	-4.15	0.1598	-3.1%	-2.2%	20	60.6%	-6.04	0.4201
Placebo	Baseline	16	128.63								
	Day 7	14	125.36	-1.93	>0.5000	-1.5%	-1.1%	7	50.0%		
	Day 30	14	120.00	-7.29	0.0501	-5.7%	-5.4%	11	78.6%		
	Day 60	14	131.14	3.86	0.1704	3.0%	3.4%	6	42.9%		
	Day 90	12	133.17	4.58	0.2007	3.6%	4.3%	4	33.3%		
	Day 120	12	138.33	9.75	0.0597	7.6%	8.2%	3	25.0%		
	Day 150	10	134.30	8.40	0.0780	6.5%	7.1%	2	20.0%		
	Day 180	9	128.78	1.89	>0.5000	1.5%	1.4%	4	44.4%		

** Significant change from baseline*
*** Significant change between treatments*

This lack of effectiveness on blood pressure may be due to the sequence of study procedures. In most cases, blood pressure was taken first followed by blood draw. The anticipation of having a blood draw may have elevated the subject's blood pressure, however, it is unknown that this was actually the case.

Borderline Hypertension and High Cholesterol Group – 3 LDL

The lipid profiles of the subjects randomized to treatment with Persimonal® Persimmon Leaf Extract showed a statistically significant reduction in the mean LDL values compared to mean baseline

Table 7. Diastolic Blood Pressure: Significant within-treatment t-test p-values are noted with an asterisk (*). Significant between-treatment ANCOVA p-values are noted with two asterisks (**).

Treatment	Evaluation	N	Treatment Mean	Mean Difference from Baseline	Within-Treatment t-test p-value	Percent Change Mean [1]	Percent Change Subject [2]	Number Improved	Percent Improved	Difference Between Treatments	Between-Treatment ANCOVA p-value
Persimonal®	Baseline	37	85.32								
	Day 7	37	86.24	0.92	>0.5000	1.1%	1.8%	19	51.4%	3.85	0.3425
	Day 30	35	83.77	-1.43	0.3816	-1.7%	-1.4%	22	62.9%	5.21	0.1074
	Day 60	35	86.23	1.03	>0.5000	1.2%	1.7%	16	45.7%	1.24	>0.5000
	Day 90	34	88.38	3.09	0.1204	3.6%	4.4%	14	41.2%	0.42	>0.5000
	Day 120	35	86.46	1.26	0.3713	1.5%	2.2%	15	42.9%	-2.24	0.2683
	Day 150	33	87.82	3.48	0.2158	4.1%	4.8%	16	48.5%	-2.02	>0.5000
	Day 180	33	86.18	1.85	0.2781	2.2%	2.7%	13	39.4%	-1.04	>0.5000
Placebo	Baseline	16	85.56								
	Day 7	14	83.21	-2.93	0.2631	-3.4%	-3.1%	8	57.1%		
	Day 30	14	79.50	-6.64	0.0346*	-7.8%	-7.3%	10	71.4%		
	Day 60	14	85.93	-0.21	>0.5000	-0,3%	0.0%	6	42.9%		
	Day 90	12	88.42	2.67	0.1211	3.1%	3.5%	3	25.0%		
	Day 120	12	89.25	3.50	0.0987	4.1%	4.4%	3	25.0%		
	Day 150	10	90.60	5.50	0.0968	6.4%	6.9%	3	30.0%		
	Day 180	9	88.78	2.89	0.3917	3.4%	3.4%	3	33.3%		

** Significant change from baseline*
*** Significant change between treatments*

Table 8. LDL: Significant within-treatment t-test p-values are noted with an asterisk (*). Significant between-treatment ANCOVA p-values are noted with two asterisks (**)

Treatment	Evaluation	N	Treatment Mean	Mean Difference from Baseline	Within-Treatment t-test p-value	Percent Change Mean [1]	Percent Change Subject [2]	Number Improved	Percent Improved	Difference Between Treatments	Between-Treatment ANCOVA p-value
Persimonal®	Baseline	36	3.63								
	Day 7	34	3.31	-0.33	0.0326*	-9.1%	-7.2%	23	67.6%	-0.14	>0.5000
	Day 30	30	3.45	-0.23	0.0538	-6.4%	-4.8%	16	53.3%	-0.05	>0.5000
	Day 60	29	3.34	-0.35	0.0485*	-9.7%	-7.6%	17	58.6%	-0.17	>0.5000
	Day 90	26	3.27	-0.32	0.0085*	-8.9%	-8.4%	18	69.2%	-0.04	>0.5000
	Day 120	24	3.46	-0.22	0.0114*	-6.1%	-5.6%	15	62.5%	0.09	0.4327
	Day 150	22	3.59	-0.13	0.2552	-3.6%	-3.2%	14	63.6%	-0.05	>0.5000
	Day 180	22	3.63	-0.08	>0.5000	-2.1%	-1.3%	12	54.5%	0.01	>0.5000
Placebo	Baseline	16	3.63								
	Day 7	13	3.42	-0.19	0.1257	-5.3%	-6.3%	7	53.8%		
	Day 30	14	3.39	-0.18	0.1947	-4.9%	-4.4%	5	35.7%		
	Day 60	14	3.39	-0.19	0.3318	-5.1%	-4.5%	7	50.0%		
	Day 90	13	3.23	-0.28	0.0655	-7.9%	-7.4%	9	69.2%		
	Day 120	9	3.22	-0.31	0.0221*	-8.6%	-8.7%	7	77.8%		
	Day 150	7	3.46	-0.09	>0.5000	-2.4%	-2.2%	4	57.1%		
	Day 180	7	3.46	-0.09	>0.5000	-2.4%	-2.1%	3	42.9%		

** Significant change from baseline*
*** Significant change between treatments*

values at the following timepoints: Day 7, Day 60, Day 90, and Day 120. Subjects randomized to treatment with the Placebo showed a statistically significant reduction in mean LDL values compared to baseline at Day 120. Between treatment analysis indicated no statistically significant differences between the two treatments at any timepoint.

Borderline Hypertension and High Cholesterol Group – 3 Total Cholesterol

The lipid profiles of the subjects randomized to treatment with Persimonal® Persimmon Leaf Extract showed a statistically significant reduction in the mean Total Cholesterol values compared to mean baseline values at the following timepoints: Day 7, Day 30, and Day 120. Subjects randomized

Table 9. Total Cholesterol: Significant within-treatment t-test p-values are noted with an asterisk (*). Significant between-treatment ANCOVA p-values are noted with two asterisks (**).

Treatment	Evaluation	N	Treatment Mean	Mean Difference from Baseline	Within-Treatment t-test p-value	Percent Change Mean [1]	Percent Change Subject [2]	Number Improved	Percent Improved	Difference Between Treatments	Between-Treatment ANCOVA p-value
Persimonal®	Baseline	37	5.78								
	Day 7	35	5.44	-0.37	0.0371*	-6.3%	-5.2%	22	62.9%	-0.22	0.4121
	Day 30	31	5.62	-0.24	0.0409*	-4.1%	-3.3%	17	54.8%	-0.07	>0.5000
	Day 60	30	5.55	-0.32	0.1025	-5.6%	-4.2%	19	63.3%	-0.34	0.2995
	Day 90	27	5.54	-0.19	0.1123	-3.3%	-3.0%	16	59.3%	0.05	>0.5000
	Day 120	25	5.57	-0.21	0.0205*	-3.6%	-3.4%	18	72.0%	0.04	>0.5000
	Day 150	23	5.69	-0.16	0.2489	-2.7%	-2.5%	16	69.6%	-0.04	>0.5000
	Day 180	23	5.81	-0.03	>0.5000	-0.6%	-0.1%	12	52.2%	0.14	>0.5000
Placebo	Baseline	16	5.76								
	Day 7	13	5.63	-0.15	0.2894	-2.5%	-2.7%	6	46.2%		
	Day 30	14	5.57	-0.16	0.2136	-2.9%	-2.6%	8	57.1%		
	Day 60	14	5.75	0.01	>0.5000	0.2%	0.5%	6	42.9%		
	Day 90	13	5.46	-0.24	0.1085	-4.1%	-3.8%	8	61.5%		
	Day 120	9	5.49	-0.24	0.0191*	-4.2%	-4.2%	8	88.9%		
	Day 150	7	5.59	-0.11	0.4758	-2.0%	-1.9%	5	71.4%		
	Day 180	7	5.53	-0.17	0.4386	-3.0%	-2.6%	4	57.1%		

** Significant change from baseline*
*** Significant change between treatments*

to treatment with the Placebo showed a statistically significant reduction in mean LDL values compared to baseline at Day 120. Between treatment analysis indicated no statistically significant differences between the two treatments at any timepoint.

Conclusion

The purpose of this study was to define whether administration of Persimonal™ daily would improve the cardiovascular system in subjects who were experiencing borderline hypertension, high total cholesterol, or high LDL levels or both high blood press and high total cholesterol and/or high LDL. The design of the clinical trial was appropriate to reveal that Persimmon Leaf Extract as a nutritional supplement ingested over 180 days was safe and efficacious in improving cardiovascular health indicators. The results of the study provide data supporting the view that Persimonal® may be administered to patients as a potential cardiovascular dietary supplement. Further research will elucidate additional benefits from this multifunctional source.

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Phytochemical-Induced Thyroid Hormones Disruption

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Abstract

Thyroid hormones (THs) are quite essential for development as well as growth. The proper release of these hormones is mandatory for the normal body functioning which may be disrupted by numerous phytochemicals that contribute to minor changes in circulating THs. Plant ingredients have attracted a lot of attention in recent decades because of their antioxidant, anti-inflammatory, antimicrobial, and anti-proliferative activities. However, there have been concerns expressed about their possible toxicity, especially when ingested in excessive doses. Some plant constituents have long been known to have anti-thyroid properties. The data mentioned in current study was obtained from various abstracting services like google scholar, pubmed and medline by using various key words such as thyroid hormones, polyphenols and THs, polyphenol induced disruptions etc. The data included in this study was mostly taken from recent research studies. In this study we conclude various polyphenols with their known mechanisms of action on thyroid cells or THs secretion and metabolism, with a special focus on experimental investigations that could have a big impact on human health as they indirectly put a negative effect on THs, deiodinase activity, TPO activity and thyroid cell growth.

Key Words: Thyroid Hormones, Polyphenols, Anti-thyroid, Deiodinase, TPO activity

Introduction

The thyroid gland which is located between C5 and T1 vertebral column is a highly vascularized gland to produce and store iodine. It produces two iodinated molecules, iodothyronines and iodotyrosines, the former including T3 and T4 hormones (A.Q Hayat, 2019) (Benvenga et al., 2018). However, thyroid stimulating hormone (TSH) is released by anterior pituitary gland which in return stimulates the release of triiodothyronine (T3- 20%), and thyroxin (T4- 80%) (Pirahanchi et al, 2021). Any disruption in the production, release or storage of thyroid hormones causes thyroid related diseases like hypothyroidism, hyperthyroidism, or thyroid cancers (Francesca Pistollato et al., 2019). Thyroid hormones are the key regulators for normal growth and development. Out of all the endocrine diseases, thyroid disorders are the most common as it gets affected by several substances capable of interfering its biosynthesis and metabolism. And many nutritional and environmental factors are capable to interfere with thyroid hormone biosynthesis and metabolism (Goncalves et al., 2017). The role of phytochemicals (naturally occurring compounds of plants) in thyroid hormone disruption is quite evident as they adversely affect thyroid function (Behl et al., 2021). For instance, the major phytochemical class called flavonoids is involved producing goiter and hypothyroidism as well as, positive effects on thyroid functions, chiefly with low iodine intake areas. Thyroid synthesis, its availability, and metabolism may also be effected by use phytochemicals (Dos Santos et al., 2011). The Consumption of flavonoids with low iodine uptake has been linked with increased risk of hypothyroidism (F Pistollato et al., 2018). Polyphenols like flavonoids and iso-flavones have the potential to produce goiter and endemic hypothyroidism. Contrarily, recent studies also suggest that plant originated flavonoids could be used in thyroid cancer treatment. (Goncalves et al., 2017). The antithyroid effect of flavonoids was first published in 1950s when Moudgal and co-workers fed rats with 20mg isolated nut pigments arachidoside and anacardioside, which produced goiter. The authors have discussed how polyphenols were proficient not only in preventing THs biosynthesis but also to reduce iodide uptake in-vitro as shown in figure 1 (Moudgal et al., 1958). This article encompasses the im-

pact of phytochemicals on thyroid hormones disruption and their possible effects in determining thyroid diseases using in-vivo and in-vitro studies. We have also discussed the mechanism of action of these plant based compounds so that it can be translated to clinical practice.

Polyphenols

Polyphenols occupy a central place in plants as major secondary metabolites and characterized by two or more phenolic groups. These compounds are actually formed by two major biochemical pathways i.e., the polyketide and shikimate pathways. However, those compounds which are constituted by one benzene ring bearing two or more –OH groups are termed as phenols instead of polyphenols (Quideau et al., 2011). Polyphenols perform various functions in plants i.e., defense against insects and microbes (Zaynab et al., 2018). Moreover, they also provide protection against solar UV rays. Depending on chemical structure the polyphenolic compounds are categorized in flavonoids, lignans, stilbens, and curcuminoids (Mondal et al., 2019).

Flavonoids

Flavonoids are polyphenolic compounds comprising of a diverse group of almost 6000 chemical compounds. As far as, their structures are concerned, these have two benzene rings in general which are attached to each other by a pyran ring. This structure can also be exhibited as C6–C3–C6. These compounds are widely found in several vegetables and fruits where they are present as the major chemical constituents. Due to their complex chemical structure, they are classified in 6 subgroups i.e., flavones, flavonols, isoflavones, anthocyanidins, flavanones and flavanols (Santhakumar et al., 2018). The impact of flavonoids on thyroid glands have been have been investigated in several experimental studies and it had been initiated about 60 years ago. Later on, coworkers investigated in is experimental study that the goiter endemia could occur due to food constituted with glycosilflavones. Recently, the already established therapeutic activities of flavonoids i.e., antimicrobial, antioxidants, antitumor and anti-inflammatory, led towards several experimental studies that prove helpful in understanding their impact on thyroid functions (Benvenga et al., 2020). Furthermore, the work of Gaitan also investigated that the anti-thyroid effect of millet glycosilflavones was associated with the retardation of TPO activity. Later on, several in vitro studies also exhibited the same mechanism while showing both competitive and non-competitive mechanisms Table 1. In addition to anti-thyroid cells effect, these naturally accuring compounds also disrupt the metabolism of THs and their actions. The in vitro data mentioned in this study was also validated in vivo, however, the results may vary depending on route, dose and models employed in during investigations. The actual impact of flavonoids on thyroid glands depends on the their amount of ingestion. The intake of flavonoids depends on eating habits of human beings i.e., the Western world consumes 20mg to 35 mg flavonides on daily basis that reaches to 500 mg if too much vegetables and fruits (Perez-Jimenez et al., 2011). The absorption of flavonoids may vary (10 to 60%) based on various factors i.e., the preparation and processing foods, liver and intestinal metabolism and the molecular structure (Goncalves et al., 2017). So, we can expect a high interindividual variation in plasma level of flavonoids after ingestion in human beings (Larson et al., 2012). Although a number of flavonoids possess anti- thyroid effect and most of them are mentioned in tables 1.

Phenolic Acid

The word "phenolic acids or phenolcarboxylic acids" are one of the main classes of polyphenols. They are abundantly found in plant-based food in bound form like seeds, fruits and vegetable leaves etc. Typically, they are present in bound from such as amides, esters, or glycosides and rarely in free form (Kumar & Goel, 2019). The phenolic Acids are distinguished mainly in two classes : Phenolic acids are mainly divided in to two classes: hydroxybenzoic acids and hydroxycinnamic acids, where the concentration of former is low in plant originated foods and fruits except some red color fruits, potatoes and onions (Lafay & Gil-Izquierdo, 2007). The hydroxcinnamic acids are cinnamic acid derivatives present in foods and frequently designated

Table 1. Showing the effects of flavonoids on thyroid hormones status. Where, THs (thyroid hormones), D1 (deiodenase-1), D2 (deiodinase-2).

Class	Compounds	Experimental model	Effects	Ref
Flavanones	Naringenin	Porcine thyroid glands	↓ Tyrosine iodination	(Goncalves et al., 2017)
	Naringin	Porcine thyroid glands	↓ Tyrosine iodination	
Flavanols	Catechin	Porcine thyroid glands	↓ Tyrosine iodination ↓ TPO activity	(Larson et al., 2012).
		Rat thyroid microsome fractions	↓ Thyroid D1 activity Ferreira	(Mondal et al., 2019).
Flavones	Apigenin	Pig thyrocytes	↓ THs synthesis	(Zaynab et al., 2018).
Isoflavones	Biochanin A	Porcine thyroid glands	↓ Iodination	(Goncalves et al., 2017)
	Genistein	Porcine thyroid glands	↓ Formation of Iodotyronine	(Pirahanchi et al, 2021).
		Human serum	↓ Thyroid hormones binding to Transthyretin	
		HEK293 cells	↓ Thyroid D1 activity	
	Daidzein	Porcine thyroid glands	↓ Tyrosine iodination	(Behl et al., 2021)
Flavonols	Quercetin	Porcine thyroid glands	↓ Tyrosine iodination	(Dos Santos et al., 2011).
		Rat thyroid microsome fractions	↓ Thyroid D1 activity	
		FRTL-5 cells	↓ Cell growth	
		RMS-13 cells	↑ D2 activity	
	Kaempferol	Porcine thyroid glands	↓ Tyrosine iodination ↓ TPO activity	(Benvenga et al., 2020).
		Rat thyroid microsome fractions	↓ Thyroid D1 activity	
		RMS-13 cells	↑ D2 activity	
		GH4C1 cells	↓ Thyroid D1 activity	
	Myricetin	Porcine thyroid glands	↓ Tyrosine iodination	(Goncalves et al., 2017).
		human thyroid glands	↓ TPO activity	
	Morin	Porcine thyroid glands	↓ Tyrosine iodination	(Santhaku mar et al., 2018).
		Rat thyroid microsome fractions	↓ Thyroid D1 activity	

as simple esters with quinic acid/glucose. Chlorogenic Acid (a combination of caffeic and quinic acids) has the highest biological activities. Other analogs are ferulic, p-coumaric, caffeic, and sinapic acids mainly found in rasberries, blue berries, coffee, cherries, apples, beans pears and white potatoes (King & Young, 1999). Similarly, four abundantly hydroxybenzoic acids are: vanilli, syringic, p-hydroxybenzoic and protocatechuic acids (Kumar & Goel, 2019). Touhami et al. studied goiter genic activity of three phenolic acids (caffeic acid, ferulic acid, p-coumaric acid). At dose 0.25 mol/kg/day, there was significant increase in thyroid weight, increased hypertrophy, decreased T3 and T4 and elevated Thyroid stimulating hormone (TSH) (Khelifi- Touhami et al., 2003).

The mode of TPO inhibition was studied in Chlorogenic acid, rosmarinic acid, quercetin, and rutin with 1C50 ranging from 0.004 mM to 1.44 mM. In this study, competitive inhibition was shown by rutin and rosmarinic acid, non-competitive by chlorogenic acid and uncompetitive inhibition by quercitin (Habza-Kowalska, Kaczor, Żuk, Matosiuk, & Gawlik-Dziki, 2019). From literature it is evident that polyphenols have vast activity on Thyroid Peroxidase (TPO). When an in-vitro and in silico screening testing was performed, it was found that Phenolic Acids (from food majorly) acti-

vated TPO. Ferulic acid (FA) effectively scavenges free radicals and inhibits lipid peroxidation. Cardio protective effects and role in tumor progression is also very evident in literature (Habza-Kowalska et al., 2021). Alomost 50 years ago, the effects of Phenolic compounds on thyroid hormone production and thyroid gland functioning were proven. However, the exact mechanism is unclear. Some phenolic compounds when consume in high doses inhibit TPO activity, as a result of which thyroid hormone synthesis is reduced as shown in table 2. A compensatory rise in TSH levels is seen in return, which causes goiter genic effects (Divi & Doerge, 1994). Even though the special effects of phenolic compounds on the thyroid gland and thyroid hormone production were proven almost 50 years ago, the exact mechanism is unclear. In the early studies on phenolic compounds by Fawcett and Kirkwood (1953) and Doniach and Fraser (1950) reported the mechanism by which resorcinol a phenolic compound causes iodination of tyrosine by TPO. As a result of which, iodotyrosines amount depletes and iodinated resorcinol. Nevertheless, some studies have also shown that there is competitive iodination of resorcinol and other phenolic compounds as the mechanism for anti-thyroid functioning (Divi & Doerge, 1994). In a study by Benorous et. al., phenolic and alkaloid extracts were studied for novel

Table 2. Effects of curcuminoids on thyroid hormones in-vivo/in-vitro analysis legends (↓) decrease/ lower, (↑) increase/rise, (-) inhibits, NaF (sodium flouride), male (M), adult (A), T3 (triiodothyronine) and T4 (tetraiodothyronine), TSH (thyroid stimulating hormone).

Class	Compound	Experimental model	Doses	Treatment duration	Effects	Ref
Phenolic acids	P-coumaric acid Ferulic acid Caffeic acid	Wistar Albino Rats (A,M)	0.25 mol/kg/d ay	3 weeks10	↑ thyroid weight ↑Hypertrophy/ hyperplasia of follicles ↑thyroid gland enlargement ↓T3 and T4 ↑TSH Thyroid lesion seen due to ↑cell growth	(Khelifi- Touhami et al., 2003)
	Chlorogenic acid Rosmarinic acid	TPO extracted from Frozen thyroid gland	0.004 mM to 1.44 mM	–	(-) of TPO activity at different potencies	(Habza- Kowalska, Kaczor, et al., 2019)
	Gallic Acid	Horseradish peroxidase invitro model	25.0 ± 4 ug/ ml	–	(-)TPO activity	(Benarous et al., 2021)
	Trans-cinnamic acids	TPO obtained from Porcine thyroid gland	0.10 ± 0.005 (AC50)	–	Dose dependent TPO activity	(Habza- Kowalska et al., 2021)
	Syringic acid	TPO obtained from Porcine thyroid gland	0.69 ± 0.034 (AC50)	–	Dose dependent TPO activity	(Habza- Kowalska et al., 2021)
	Ferulic acid	TPO obtained from Porcine thyroid gland	0.39 ±0.019 (AC50)	–	Dose dependent TPO activity	(Habza- Kowalska et al., 2021)
	Sinapic acid	TPO extracted from porcine thyroid glands	25.42 ± 1.13 ug/ml (EFC50)	–	Competitive TPO activity Inhibitor	(Habza- Kowalska, Gawlik- Dziki, & Dziki, 2019)
	p-coumaric acid	Female adult rats of Wistar rats	3 ng/µl	–	Disrupts thyroid hormone biosynthetic pathway	(Sarkar et al., 2021)

peroxidase inhibitor activity. Gallic acid inhibited the TPO activity at 25.0 ± 4 ug/ml effectively (Benarous et al., 2021).

Curcuminoids

Curcuminoids are the polyphenols that belong to curcuma species and are extracted from the roots of *C. longa* (Turmeric). It constitutes about 3-5 % of total curcuminoids. Main derivatives are Curcumin, bis-demethoxycurcumin (BSDMC) and demethoxycurcumin (DMC), tetrahydrocurcumin (THC) & turmerones. Curcumin; also called [diferuloylmethane, 1,7-bis-(4- hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione] is a yellow colored phytochemical which is abundantly studied because of its vast biological properties as compared to other derivatives isolated (Itokawa et al., 2008). Curcumin exhibits Antimicrobial, antiviral, anti-inflammatory and antioxidant activities. As far as its therapeutic value is concerned, curcumin shows immunomodulatory, cytotoxic, Beside these, curcumin has a variety of potentially therapeutic properties, such as antineoplastic, antiapoptotic, antiangiogenic, antidiabetic activities (Cikrikci et al., 2008). Curcumin analogs, when investigated, have shown cell proliferative, inflammatory and thyroid disrupting activity as shown in table 3 (Al-Suhaimi et al., 2011). Curcumin at 100mg/kg had increased FT3 and FT4 levels in 3 month old wistar rats when given for 7 days. it has also reported decreased levels of FT3 with the same dose and duration of treatment in 18 month old wistar rats concluding the effects of aging on treatment (Papiez et al., 2008). Curcumin was designated as Endocrine disrupter when at 10*ADI *(Admissible daily intake of 38.50 mg/kg/body wt.), it affected the thyroid functioning potentially producing adverse effects (Shakoor et al., 2022). Similar effects were seen when turmeric extract obtained from *C.longa,* was given to male wistar rats for 15 days. serum T4 and T3 levels in the blood fluctuated from normal and ultimately rise in their level caused thyroid hormone dysfunctioning.

Table 3. Showing the effects of curcuminoids on thyroid hormones where (↓) decrease/lower, (↑) increase/rise, (-) inhibits, NaF (sodium fluoride), male (M), adult (A), TSH (thyroid stimulating hormone), T3 (triiodothyronine), T4 (tetraiodothyronine).

Class	Compound	Experimental model	Dose	Treatment duration	Effect	Ref
Curcuminoids	Curcumin	Sprague Dawley albino rats (M,A)	NaF+Curcumin-100/mg/kg/day	2 weeks	↓serum T3 and T4 levels ↑TSH level	(Abdelaleem et al., 2018)
	Turmeric extract	Adult Male Wister rats	1% extract in 20mg protein diet	15days	↑ T4 & T3 levels in blood	(Peepre, Deshpandey, & Choudhary, 2014)
	Curcumin	Albino Rats	admissible daily intake (ADI) and 10× ADI= 3.85 and 38.5 mg/kg/body wt.	15days	Disturbed thyroid functioning at mentione d dose.	(Shakoor et al., 2022)
	Curcumin	3 month old Wistar rats (M)	100 mg/kg	30 days	↑FT3 and FT4 level	(Papiez et al., 2008)
	Curcumin	18-month- old male Wistar rats	100 mg/kg	30 days	↓FT3 level ↑ % of larger follicles	(Papiez et al., 2008)
	Curcumin	Rats (M)	200 mg/kg	25 days	↑ thyroid hormone in diabetic rats,	(Sadoughi & Edalatmanes h, 2017)

Lignans

Lignans are naturally occurring diverse class of secondary metabolites constituted by phenylpropanoid core. These compounds are present in various plant-based foods i.e., whole grains, seeds, fruit, legumes and vegetables (Rodríguez-García et al., 2019). These compounds have various therapeutic activities i.e., antioxidant, anticancer, estrogenic, and antiestrogenic properties (Durazzo et al., 2018). Epidemiological studies have shown that lignans intake is linked with a reduction of thyroid cancer occurrence owing to their impact on estrogen metabolism (Horn- Ross et al., 2002). However, no specific data is still available that can elaborate their impact on normal thyroid function and growth except the antagonistic role of (+) pinoresinol and (−) arctigenin on human TH receptor β in a cell-based bioassay. The study exhibited that (+) pinoresinol and (−) arctigenin had IC50 of 8.2 and 3.8 μM respectively (Ogungbe et al., 2014). However, this antagonistic effect is still doubtful whether it is of clinical relevance or not (Cheng et al., 2010).

Alkaloids

Phytochemicals have grown in popularity around the world as a means of enhancing health and disease prevention. Alkaloids are essential chemical substances that can be used to develop new drugs (Shi et al., 2014). These are the secondary metabolites of the plants that serve to protect plants from predators and bacteria, as well as repel microbes and herbivores, depending on the toxicity of the microbes as well as modulate various clinical illnesses in human beings (Wink & Schafer, 2009). These chemical entities have the potential to be used as immunomodulatory, anticarcinogenic, antiaging and antimicrobial agents, and they are divided into several classes based on their structure, including quinolizidines, pyrrolidines, indoles, pyrrolizidines, tropanes, purines, imidazoles, piperidines, and isoquinolines (Thawabteh et al., 2019). Along with these potential therapeutic actions of alkaloids, they are widely involved in the hormonal imbalance in human beings including pituitary hormones, thyroid hormones, steroidal hormones as shown in table 4 (Delitala et al., 1983; Shekar-Foroosh et al., 2012).

Arecoline is a naturally occurring hallucinogenic alkaloid found in Areca catechu's betel nut. It is a partial agonist of the nicotinic and muscarinic acetylcholine receptors and has a variety of pharmacological effects, involving endocrine as well as metabolic effects. Thousands of people chew it to boost work capacity and relieve stress (Volgin et al., 2019). Arecoline has a twofold effect on the thyroid gland of mice, stimulating thyroid function at first and then inhibiting thyroid activity, most likely due to the cytotoxic effect of this substance, as seen by ultrastructural changes in thyrocytes (Dasgupta et al., 2010). In adult male mice, acute administration to arecoline elicited a rise in blood T3 and T4 levels as well as a decrease in serum TSH levels. Arecoline therapy has also been demonstrated to exacerbate hypothyroidism in mice under metabolic stress (Dasgupta et al., 2017). Similarly, nicotine is also a well-known alkaloid that is a major component of tobacco. Nicotine can also be consumed through chewing tobacco leaves or swallowing pills, in addition to smoking. Nicotine has been demonstrated in several studies to impair thyroid function, particularly in the early stages of development (Lisboa et al., 2015). When nicotine is delivered to the C57BL/6 mice model at doses of 24 mg/kg BW by s.c. minipump concentration for 12 days, it has different effects such as a reduction of serum T4 concentration and an elevation in the T3/T4 ratio after 24 hours of nicotine withdrawal (Leach et al., 2014).

Harmine, is found in a variety of medicinal plants, has an IC50 of 141.4 M and inhibits horseradish peroxidase activity. Molecular modelling revealed that same information also applies to TPO, implying that it might be used as an anti-thyroid medication because of their inhibitory effect on peroxidase activity which ultimately increase T3 and T4 level (Benarous et al., 2021). In Swiss albino mice, piperine, the main alkaloid found in Piper nigrum (black pepper), drastically reduced serum T3 and T4 concentrations, as well as liver D1 activity (Panda & Kar, 2003). The mice were given 2.5 mg/kg/day and the outcomes were evaluated. Only liver D1 activity and serum T3 concentrations were reduced at a lower dose of 0.25 mg/kg/day. However, because black pepper comprises about 5–9% piperine, these levels are significantly below those found in human nutrition (Dudhatra et al., 2012; Vijayakumar & Nalini, 2006).

Table 4. Exploring the effects of alkaloids on thyroid hormones. Where, T3 (tri-iodothyronine), T4 (tetraiodothyronine), TSH (thyroid stimulating hormone).

Class	Compounds	Experimental Model	Effects	Ref
Alkloids	Arecoline	Mices	Rise in blood T3 and T4 levels and lowers serum TSH levels	(Dasgupta et al., 2017)
	Nicotine	Wistar Rats	Lowers serum T4 concentration and an rise in the T3/T4 ratio	(Leach et al., 2014)
	Harmine	In-vitro assay	Inhibit the peroxidae activity and increase thyroid hormone level	(Benarous et al., 2021)
	Piperine	Mices	Reduced serum T3 and T4 concentrations	(Panda & Kar, 2003)

Conclusion

Phytochemical induced thyroid disruption is an area of great concern in modern age due to the wide exposure of phytochemicals in human body. Among phytochemicals, the polyphenols have great impact on thyroid glands. These chemical compounds disrupt the normal levels of circulating thyroid hormones. The phytochemical induced thyroid hormones disruption is accompanied by various mechanism of actions which are being investigated by various researchers and now it has become a major area of research.

Limitations

This study lacks data elaborating the impact of polyphenols in human and especially in children. So, there is a major research gap on this aspect that can further open the door for other researchers.

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Cut-off values of major parameters for evaluation of kidney function in Multiple Myeloma

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Abstract

Aim: Our aim was to evaluate sensitivity and specificity of major parameters of kidney function in risk prediction of kidney damage in patients with multiple myeloma (MM) calculating cut-off values for further screening surveys.

Methods: We analysed blood samples of 62 patients with MM and then constructed ROC curves in relation whether they had kidney injury or not. We then compared their respective areas under the curve (AUC) and determined cutoff values for grouping the patients according to risk.

Results: We found that Cystatin C with cutoff value of 1.04 mg/l and closely followed by the urinary kappa chains level with cutoff value of 0.03 g/L and AUC of 0.8, is the most significant predictor of kidney injury. β2 microglobulin cut-off value of 3.445 ml/L compared to creatinine cut-off value of 81.5 mmol/L and (AUC 0.72 vs 0.748) carried similar risk. Other parameters had cut-off values and AUC-s as follows: leukocytes (4.97; 10^9/L and AUC 0.664), neutrophile fraction (42.5 %/L and AUC 0.722), Serum kappa chains (4.66 g/L and AUC 0.629), serum kappa/lambda chain ratio (2.45 g/L and AUC 0.653) and IL-6 (5.55 pg/mL and AUC 0.625).

Conclusion: This new cut-off values could lead to early assessment of renal impairment in patients with multiple myeloma and that way improve preservation of renal function longer.

Key words: acute renal injury, biomarkers, urinary kappa chains, multiple myeloma.

Introduction

Multiple myeloma is neoplastic disorder in which monoclonal plasma cells proliferate in bone marrow producing excessive amount of immunoglobulins. Enlarged number of plasma cells in bone marrow suppress other cells and cause different cellular immune profiling that is used in prediction of MM prognosis. The approaches necessary to perform a comprehensive evaluation to the prognosis of kidney injury in MM patients include follow up of kidney injury using specific parameters.

Toxic nephropathy originating from renal or systemic disease (e.g. multiple myeloma) can be drastically worsen by additional conditions (e.g. dehydration, hypoxia), as well as by the effects of potentially nephrotoxic substances (e.g., drugs, contrast media) administrated in the course of disease (1). MM with renal impairment at presentation should be considered a medical emergency since the recovery of renal function is associated with survival benefit (2, 3) p=0.046. The risk of progression to severe organ impairment leading to renal replacement therapy is considerable, thus, it is mandatory to identify patients at risk for kidney damage at a very early stage and to institute treatment promptly.

The standard assessment of renal function in patients with MM includes creatinine and creatinine clearance, although both measurements probably underestimate the prevalence of renal dysfunction, because of the additional tubular secretion of creatinine and its dependence on extra-renal factors. Serum creatinine is a retrospective, insensitive and even deceptive measure of kidney injury. Retrospective because its concentration may result in a

very delayed signal even after considerable kidney injury (4)but no widely accepted definition exists. A recent classification system by the Acute Dialysis Quality Initiative (RIFLE. Insensitive because as much as a 50% loss of renal function may be required to elevate serum creatinine enough that it comes to medical attention. Deceptive because serum creatinine level often reflects transient physiological adaptations to volume changes or the presence of chronic kidney disease - CKD rather than the development of acute kidney injury. (5)

Terpos E et al. supporting the use of the chronic kidney disease epidemiology collaboration Cystatin C-based equation that detect more MM patients with kidney failure than equations based only on serum creatinine. It is not surprising that the diagnosis of a paraproteinemic renal lesion is hampered by the general lack of sensitivity and specificity of currently available noninvasive tests (6)such as the Modification of Diet in Renal Disease (MDRD. The measurement of serum concentration of the clonal free light chains (FLCs) and that of the urine level of albumin may be considered the landmark for screening algorithms in patients with cast nephropathy (7).

The optimal cut-off value for chosen parametars for kidney injury in MM patients has not yet been reported.

The aim of this study is to determine cut-off values of major parameters of kidney function in risk prediction of kidney damage in patients with multiple myeloma (MM) calculating cut-off values for further screening surveys.

Material and Methods

Sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), and accuracy were estimated for urinary and serum κ, ratio between urinary and serum κ and λ chains, vascular endothelial growth factor – VEGF, Interleukin 6 – IL-6, total number of leukocytes, neutrophils and β2-microglobulin, on the basis of both the central 95% interval and a diagnostic range that captured 100% of test data. Accuracy was calculated as the proportion of individuals classified correctly. Confidence intervals were calculated according to the exact binomial distribution for sensitivity, specificity and accuracy and by bootstrap for PPV and NPV.

The choice of a good cut-off value is important because it should not be too high or too low. A low cut-off value gives many false-positive results and leads to an excessive diagnosis of renal failure, especially as the prognosis value of an early renal failure is not clearly known. On the other hand, a high cut-off value will give many false-negative results and leads to an underestimated prevalence of early renal failure, which in turn will decrease the benefits of an early intervention in those patients whose diagnosis is missed.

In the context of renal failure screening, the problem is to transform a quantitative continuous variable into a quantitative binary variable (pathological value yes/no) and then to define a cut-off value for testing parameters with the best diagnostic value of renal failure. To identify the suitable value, receiver operating curves (ROC curves) were constructed.

The advantage of constructing a ROC curve is to adjust the cut-off value for serum creatinine to a specific diagnostic purpose.

As the cut-off point changed, the sensitivity and specificity of the test changed in opposite direction. The upper left-hand corner of the ROC graph denoted a perfect diagnostic test: a true positive rate of 100% and false positive rate of 0%. Thus, the point where the ROC curve was close to the left and top boundaries of the ROC graph corresponded to the value in which the sum of the specificity and sensitivity is the highest. The Younden index, defined as (sensitivity + specificity) – 1, gave the same weight to false positives as it did to the false negatives.

Subjects

62 patients with multiple myeloma (MM) entered the study. Patients with multiple myeloma were divided into 3 groups based on the stage of the disease; 21 patients with MM at presentation, 21 patients with MM in “steady“ phase of disease and 20 patients with MM in relapse phase. In each group, there were similar number of male and female patients.

Methods

Peripheral blood, i.e. serum collected from patients by venipuncture process, was used as a starting sample. Creatinine (45-115 μmol/L), β2 microglobulin (1.2-2.5 mg/L) concentrations, number of

leukocytes (4.00 – 10.0 (10^9/L)) and neutrophils (47.0 – 76.0 %L) were determined using ARCHITEKTc Systems, Abbott Diagnostics. The method is based on the fact that a strong base like NaOH reacts with creatinine to form a red chromophore. The degree of increase in absorption at 510 nm as a consequence of the formation of this chromophore is directly proportional to the concentration of creatinine in the sample and was measured at a wavelength of 510 nm.

ELISA test is an enzyme-linked immunosorbent assay that determines the presence and amount of antigen or antibody in the presence of enzymes as an indicator, and because it can detect very low concentrations of the target, substance is considered one of the most commonly used and powerful laboratory techniques. Immunological tests use a specific antibody or immunoglobulin to detect antigen. Monoclonal antibodies react with one specific, and polyclonal with several different epitopes on the antigen molecule. This test served to determine the concentration of cystatin C (0.50 – 0.96 mg/L) and concentration of VEGF (0.5–1.5 pg/mL) and IL-6 (0 - 43.5 pg/ml) in samples.

Nephelometry is a modification of photo-optical end-point detection in which 90-degree or forward-angle light scatter, rather than optical density, is measured. A light-emitting diode produces incident light at approximately 600 nm, and a photodetector detects variations in light scatter at 90 degrees (side scatter) and 180 degrees (forward-angle scatter) used for measuring sera immunoglobulin light chains – λ (0.9 – 2.1 g/L), immunoglobulin heavy κ chains (1.7 – 3.7 g/L) and κ/λ–light chain ratio (0.75 – 4.5). The normal ranges for free light chains are generally: 3.3 to 19.4 milligrams per liter (mg/L) kappa free light chains. 5.71 to 26.3 mg/L lambda free light chains. 0.26 to 1.65 ratio of kappa/lambda.

Statistical analysis of data was done using computer SPSS - Statistical package for social sciences - programs, version 26.0.

Results

For each chosen cut-off value, the sensitivity, specificity and positive and negative likehood ratios of the test were calculated. As well as the corresponding standard error. The sensitivity of the test corresponded to the rate of true positives and the specificity to the rate of the true negatives. The positive likelihood ratio was the ratio of the rate of the true positives and the rate of the false positives. The negative likelihood ratio was the ratio of the rate of the false negatives and the rate of the true negatives. A positive likelihood ratio equal to 10 means that a positive response of the test (parameter value higher than the cut-off value) is 10-fold more frequent among patients with renal failure than among the patients without renal failure. A negative likelihood ratio equal to 0.10 means that a negative response of the test (parameter lower than the cut-off value) is 10-fold less frequent among patients with renal failure than among the patients without renal failure. Cut-off values for each tested parameter are presented in Table 2.

The diagnostic function of a biological test is to evaluate, according to its results, the probability for a given disease or the probability of its absence. This function of the test can be expressed by the intrinsic qualities of the test, which are sensitivity and specificity.

The advantage of constructing a ROC curve is to adjust the sensitivity or the specificity. In the low-prevalence situation, improving the specificity will decrease the number of false positives without greatly increasing the false negatives. To determent of sensitivity, the specificity of 98% is chosen. That is why for each study, the trade-off between sensitivity and specificity has to be determined by the group of experts according to the aim(s) of the study and the use of these serum parameters cut – off values. The additional advantage of constructing a ROC curve is to adjust the cut-off values for serum parameters for a specific diagnostic purpose.

To reduce an entire ROC curve to a single quantitative index of diagnostic accuracy, the area under the curve (AUC) was calculated.

Predictive values both positive and negative were set of based on calculated sensitivity and specificity in ROC curves (Table 2.).

Further on, we examined the importance of sensitivity and specificity of renal function parameters and inflammatory markers in the prediction of renal damage in patients with multiple myeloma.

The sensitivity of urinary κ chains in the prediction of renal impairment in patients with multiple myeloma was 74.1% and specificity 80.0%, while

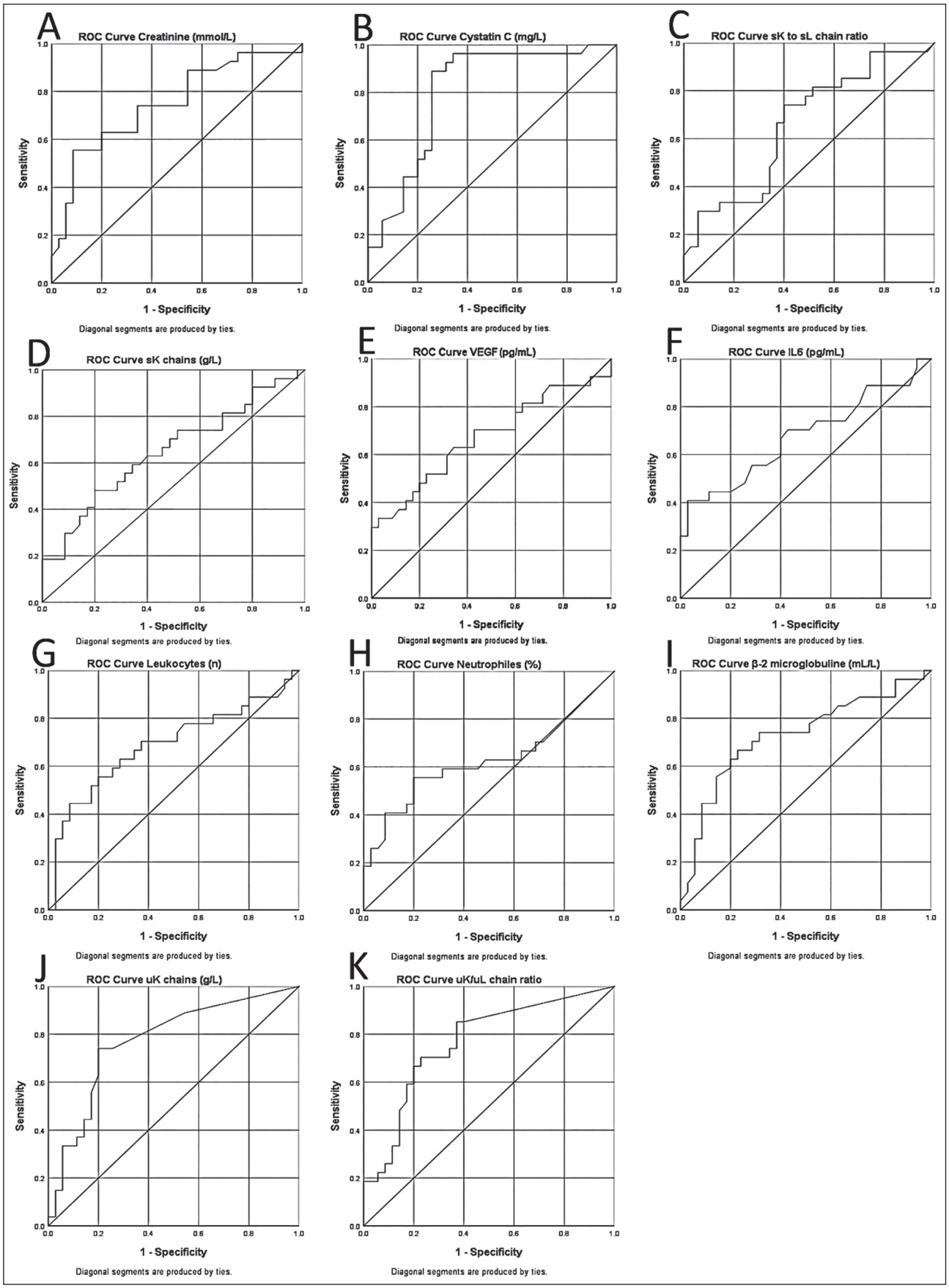

Figure 1. ROC curves for each assessed parameter. Description is given in Table 1. and 2. Cystatin C had had largest area under the curve, closely followed by urinary kappa chains (Table 1.)

the area below the ROC curve was 0.8 g/L and proved to be statistically significant (p <0.001); positive predictive value was 74.1, while the negative predictive value was 80. For the ratio between urinary κ/λ chains as the parameter in the prediction of renal impairment in MM, the sensitivity was 74.1% and specificity 65.7%; the area below the ROC curve was 0.776 g/L, that is statistically significant (p=0.002); while positive predictive value was 62.5, and negative predictive value was 76.7. For neutrophils as the parameter of renal impairment the sensitivity was 92.6, specificity 22.9, AUC 0.72 (%//L); PPV 48.1¸ NPV 80; p=0.164. For β2 microglobulin as parameter of renal impairment in MM, sensitivity was 74.1, specificity 68.6; AUC 0.72 mg/L; PPV 64.5; NPV 73.3; p=0.002. When leukocytes were checked as the parameter, the following values were achieved: sensitivity 70.4, specificity 62.9; AUC 0.664 (10^9/L); PPV 59.4; NPV 73.3; P=0.012. For serum κ/λ ratio as the parameter of renal impairment the sensitivity was 74.1, specificity was 60; AUC 0.653 mg/L; PPV 58.8; NPV 75; p= 0.01 while for serum κ chains sensitivity was 63, specificity 60; AUC 0.629 mg/L; PPV 54.8; NPV 67.7; p=0.124. Vascular endothelial growth factor (VEGF) and Interleukin 6 (IL-6) as angiogenic factor and proinflammatory cytokine had almost identical values as predictors of renal impairment in MM; sensitivity: 70.4, 70.4; specificity: 57.1, 57.1; AUC 0.651 pg/mL, 0.625 pg/mL; PPV 55.9, 55.9; NPV 71.4, 71.4; p=0.041, p=0.041, respectively. Finally, we constructed ROC curves for creatinine and Cystatin C as known predictors of renal impairment with or without MM and achieved following values: sensitivity: 74.1; 88.9; specificity: 65.7; 74.3; AUC: 0.748mmol/L; 0.803 mg/L; PPV: 62.6; 72.7; NPV: 76.7; 89.7; with p equal to 0.002 and <0.001, respectively.

The results indicated that reduction in glomerular filtration rate (as evidenced by rising serum creatinine and Cystatin C concentrations) resulted in elevation of both FLC, without alteration of the κ/λ ratio. β2 microglobulin retains an important role in providing diagnostic information and enabling the comparison of disease staging between different studies. Monitoring the κ/λ ratio was also a useful means of distinguishing the effect of renal damage from tumor growth (8) and in our patients with MM this urinary κ/λ ratio values had statistical significance of 0.002.

In study of multiple myeloma that performed Mead et al., there was not always acute impairment of renal function, but also chronic renal impairment, which occurs as a result of the production of a certain concentration of paraproteins that in the process of catabolization lead to the formation of κ or λ free light chains leading to "cast" formations, which is the basis of impaired renal function in multiple myeloma (9).

Table 1. Cut-off values, sensitivity, specificity, positive /negative predictive value, area under the curve and 95% confidence interval for major parameters.

	AUC	95%CI	Cut-off value	Se	Sp	PPV	NPV	p-value
Cystatin C	0.803	(0.639-0.931)	1.04	88.9	74.3	72.7	89.7	<0.001
Urinary K	0.8	0.636-0.925	0.03	74.1	80	74.1	80	<0.001
Urinary K/λ ratio	0.776	0.606-0.909	0.15	74.1	65.7	62.5	76.7	0,002
Creatinine	0.748	0.667-0.952	81.5	74.1	65.7	62.5	76.7	0.002
Neutrophils	0.722	0.574-0.897	42.5	92.6	22.9	48.1	80	0,164
β²microglobulin	0.72	0.570-0.889	3.445	74.1	68.6	64.5	77.4	0,002
Leukocytes	0.664	0.462-0.813	4.97	70.4	62.9	59.4	73.3	0,012
Serum k/λ ratio	0.653	0.450-0.804	2.45	74.1	60	58.8	75	0,01
VEGF	0.651	0.424-0.791	125.5	70.4	57.1	55.9	71.4	0,041
Serum K chains	0.629	0.439-0.790	4.66	63	60	54,8	67.7	0,124
IL6	0.625	0.476-0.828	5.55	70.4	57.1	55.9	71.4	0,041

AUC – Area Under the Curve; 95% CI- 95% confidence interval; Se-sensitivity; Sp- specificity; PPV-Positive Predictive Value; NPV- Negative Predictive Value; p<0,05 was considered statistical significance

**We did not report parameters whose area under the curve was below 0.625.*

Close associations between Cystatin C, serum creatinine and β2 M have also been reported (10, 11, 12).

Recent studies have shown that serum cystatin C is superior to creatinine in the assessment of early renal impairment, including patients with multiple myeloma. Elevated Cystatin C levels were observed in 57.3% of newly diagnosed patients with multiple myeloma, while high serum creatinine levels were detected in only 23.5% of patients (13).

The incidence of renal impairment in multiple myeloma varies from study to study. This is primarily caused by the different definition of renal impairment in each study.

In a study conducted by Murty et al., (13) serum creatinine and Cystatin C were studied and analyzed for the occurrence of early renal impairment, and the study included two groups of patients, patients with acute renal impairment and a control group. Serum Cystatin C had a lower standard deviation (SD = 1.1), while serum creatinine had a higher (SD = 1.8) in acute renal impairment, indicating less variability in serum Cystatin C. The variation in serum creatinine concentration was significantly higher in relative to serum Cystatin C concentration in both groups. The standard deviation of serum creatinine (SD = 0.23) was twice that of Cystatin C (SD = 0.12) in the control group, indicating a wide fluctuation of serum creatinine compared to serum Cystatin C in a healthy population. Although the correlation between these two parameters was significant in both groups, they emphasize the correlation observed in the group of patients with renal impairment. This implies that small changes in serum creatinine are best reflected by a proportional increase in serum Cystatin C in acute injury of renal function, especially at lower values. Murty et al. (13) also found that 56.2% of patients with impaired renal function had normal serum creatinine levels in the early stages of the disease, while all patients had elevated Cystatin C levels at the same time. This confirms the conclusion that serum Cystatin C is significantly elevated before serum creatinine levels begin to increase, thus aiding in the early detection of renal dysfunction (14). We came to the same conclusion in our study, where cystatin C proved to be by far the most sensitive marker in the assessment of renal injury in MM.

Our results show that only neutrophils and cystatin C had sensitivity of 92.6 and 88.9, while specificity was 22.9 for neutrophils and 74.3 for cystatin C – that pushed him up as a marker of renal impairment in patients with multiple myeloma. The sensitivity of urinary κ chains as well as urinary κ/λ chains ratio in the prediction of renal impairment was 74.1% and the specificity was 80%, and 65.7%, respectively, with an area under the curve (AUC) of 0.8 and 0.776 showed statistical significance with $p<0.001$ and $p=0.002$, respectively. $\beta^{۲}$ microglobulin has sensitivity of 74.1 and statistical significance for evaluation of renal impairment, with $p=0.002$, but it could interfere with its role in multiple myeloma classification – where is one of the major biochemical parameters, since β2 microglobulin has specificity of 68.6. This is in accordance with studies of Puthiyottil D. et al and Yue L. et al. (14-16) The association between elevated markers of inflammation and the diagnosis of multiple myeloma was also demonstrated in a study conducted by Koshiaris et al., (16) where the CRP value was statistically significantly elevated ($p = 0.005$) even before diagnosis was made. Their goal of the study was to identify blood tests that could be helpful in establishing a diagnosis of multiple myeloma. In the mentioned study, the sensitivity of CRP was 46% and the specificity 63%, which was not enough to show statistically significant sign and make it a significant predictor of the disease (16). Plasma viscosity and erythrocyte sedimentation rate have shown to be much more important markers in disease detection and exclusion, while CRP has shown to be more important in prognosis than in disease diagnosis (17).

Conclusion

Serum Cystatin C is significantly elevated before serum creatinine levels begin to increase, thus aiding in the early detection of renal dysfunction. Statistical significance is present for values of urinary κ chains as well as for values of urinary κ/λ chains. β2 microglobulin has statistical significance for evaluation of renal impairment, but it could interfere with its role in multiple myeloma classification – where is one of the major biochemical parameters, since β2 microglobulin has specificity of 68.6. Cystatin C with cutoff value

of 1.04 mg/l and closely followed by the urinary kappa chains level with cutoff value of 0.03 g/L and AUC of 0.8, is the most significant predictor of kidney injury. β2 microglobulin cut-off value of 3.445 ml/L compared to creatinine cut-off value of 81.5 mmol/L and (AUC 0.72 vs 0.748) carried similar risk. Other parameters had cut-off values and AUC-s as follows: leukocytes (4.97; 10^9/L and AUC 0.664), neutrophile fraction (42.5 %/L and AUC 0.722), serum kappa chains (4.66 g/L and AUC 0.629), serum kappa/lambda chain ratio (2.45 g/L and AUC 0.653) and IL-6 (5.55 pg/mL and AUC 0.625), All other tested parameters had lower specificity and no statistical significance in evaluation of renal impairment in patients with multiple myeloma. This new cut-off values could lead to early assessment of renal impairment in patients with multiple myeloma and that way improve preservation of renal function longer.

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Effect of Beta-Glucan on the Improvement of Immunity in Healthy Individuals during the Flu Season: A Pilot, Prospective and Open-label Clinical Trial

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Abstract

Beta-Glucans are heterogeneous polysaccharides of glucose polymer, and its activity depends on the molecular structure, size, branching frequency, structural modification, conformation, and solubility. Recent findings indicate that β-glucans enhance the immune system underlying the activation of lymphocytes, monocytes, macrophages, granulocytes, and natural killer (NK) cells. This study aims to evaluate the effect of yeast (1,3)-(1,6)-beta-glucan with 90-day consumption on the improvement of immunity in healthy individuals during the flu season. 12 healthy participants received oral yeast (1,3)-(1,6)-beta- D-glucan dose (30 mg) per day over a course of 90 days. The subjects were examined by the investigator at the study visits of enrollment (baseline), 30-day, 60-day and 90-day. Serum biomarkers and Peripheral Blood Mononuclear Cells (PBMC)s were measured at baseline, 30-day, 60-day and 90-day visits. In the study population, supplementation with yeast (1,3)-(1,6)-beta-glucan reduced the number of symptomatic upper respiratory tract infections (URTIs) by 44% as compared to the same winter time period (December through March) from the previous year ($p = 0.027$). The duration of sickness of URTIs reduced by 50% as compared to the previous year ($p < 0.0001$). The serum levels of IFN-γ, IL-2 and IFN-γ/IL-4 ratio statistically increased as compared to the baseline. The cell numbers from in vitro PBMC Cell proliferation assay for IFN-γ and IFN-γ/IL-4 ratio statistically increased. The findings from the present study suggest that yeast beta-glucan dietary supplement preparation reduced the upper respiratory tract infection by improving the body's immunity to defend pathogens.

Key words: *dietary supplement, efficacy, immune system, beta-glucan, PureMune*

Introduction

Beta-glucans are mainly found in the extracts of some species of mushrooms and in microbes, such as black yeast, and possess some unique immunological activities (1, 2). Beta-Glucans are heterogeneous polysaccharides of glucose polymer, consisting of a backbone of beta-(1-3)-linked beta -D-glucopyranosyl units with beta-(1-6)-linked side chains of varying distribution and length. The activity of beta-glucan depends on the molecular structure, size, branching frequency, structural modification, conformation, and solubility. It appears that the most active forms of β- glucans contain beta-(1-3)(1-6) linkages (3). Beta-glucans have been shown to exert cytotoxic activity against cancer cells (4) accompanied by activating the production of interleukin-2 (IL-2), IL-4, IL-6, IL-12, CD44, interferon-γ (IFN-γ) and tumor necrosis factor-α (TNF-α) (5,6). These findings indicate that β-glucans enhance the immune system underlying the activation of lymphocytes, monocytes, macrophages, granulocytes, and natural killer (NK) cells (7). Beta- Glucan has been shown to protect against infection by bacteria, viruses, and pathogenic microorganisms (8).

Beta-Glucan also prevents cancer promotion and progression and has synergistic anti-tumor effects with monoclonal antibodies and cancer chemotherapeutics (9). Beta-Glucan promotes antibody- dependent cellular cytotoxicity through a biological pathway involved in carcinogenesis (10). In general, the anticancer actions of β-glucans are not attributable to their direct actions on cancer cells, as is the case with chemical anti-cancer drugs, but depends on the immunological enhancement of the host, e.g., by acting as a biological response modifier (BRM) (4).

Macrophages and dendritic cells have typical cell surface receptors called pattern recognition receptors (PRRs) that detect innately non-self-molecules including pathogen-associated molecular patterns (PAMPs) (11). β-Glucans might act as PAMPs and are recognized by PRRs, because β-glucans cannot directly penetrate cell membrane due to their large molecular size (12). The major PRRs for β-glucans might be dectin-1 and the roll-like receptor (TLR). After binding with β- glucan, dectin-1 and TLR may induce signaling cascade and activate immune cells. Other receptors, such as complement receptor 3 (CR3), scavenge receptors (SR), and lactosylceramide (LacCer), may be involved as well (11, 13).

The biological effects beta-1,3/1,6-glucans depends on the interaction between beta-1,3/ 1,6-glucan and specific receptors on epithelial surfaces (14). However, beta-1,3/1,6-glucans may stimulate the gut immune system, such as suppressive effects on asthma and allergy symptoms. Beta-1,3/1,6-glucan may interact indirectly with the gut microbiota by affecting intestinal barrier function and LPS toxicity, and by enhancing the production and secretion of components such as lysozyme, antimicrobial peptides and IgA.

Several double-blind, randomized and controlled clinical studies have demonstrated that beta- glucans could reduce the incidence of common cold, upper respiratory infection and improve the mood state in young children, adults and stressed women (15, 16, 17, 18).

This study is designed to examine the effect of PureMune with beta-glucan on the improvement of immunity in healthy individuals during the flu season. The immunity can be measured by analyzing the immune biomarkers of IFN γ, IL-2, TNF-alpha/beta, IL-4, IL-5, IL-10, IL-13, IL- 12, IL 17, IL-6 and CD44 at baseline, 30-day, 60-day and 90-day visits. In addition, in vitro cell proliferation assay of PBMCs can be measured to assess immune cells DNA synthesis at baseline, 30-day, 60-day and 90-day visits.

Methods

Study population and investigational product

Twelve subjects with at least four common colds within the last twelve months were enrolled into the study. They must meet the following inclusion criteria: written consent to participate, age ≥ 18–80 years, at least four common cold infections within the last 12 months and agree not to take any nutritional medications or supplements during the study. The main exclusion criteria were as follows: diseases of cardiovascular, cerebrovascular, liver, kidney, hematopoietic system and other serious diseases, congenital or acquired immunodeficiency diseases, pregnant or lactating, has an allergy to beta-glucan, takes any drugs or food supplement related to the study product in recent days, participated another clinical trial in the past three months, has any diseases or takes any drugs or nutrition products that can affect the evaluation of the study product.

The beta-glucan is an insoluble (1,3)-(1-6)-beta glucan which was made from Baker's yeast (S. cerevisiae), with a purity of at least 80% on dry matter (branching factor approximately: 1,3[backbone]: 1,6 [side chain]: 1,3/1,6 [branching] = 10:1:0.6). In addition, it contains <2% α-D-Mannose; < 6% fat; < 3% protein; <6% moisture; and < 3% ash on dry matter. The dry matter is more than 80%.

Subjects received a total of 30 mg of insoluble yeast beta-glucan (PureMune provided by ImmuDyne Nutritional LLC, USA) in one sachet bag per day for 90 days.

The clinical study was approved by the Ethics Committee of Shanghai Nutrition Society. The written informed consent was obtained from all participants prior to entering the study. The study was registered in the Chinese Clinical Trial Registry at https://www.chictr.org.cn/index.aspx ChiCTR2000040893.

Study design

The study was a single center, open label, prospective pilot trial which was carried out in accordance with the Helsinki declaration and ICH GCP E6 from December 2020 to April 2021 in the Department of Gastroenterology of Jinhua People's Hospital, Jinhua, Zhejiang Province, China.

The eligible twelve subjects were enrolled at one study site. There were four study visits of baseline, 30-day, 60-day and 90-day during the study period of 12 weeks. A common cold episode was defined by the occurrence of at least two of the following cold symptoms: fever, cough, runny nose and throat pain. During the common cold, the subjects were instructed to record and assess their cold symptoms at home for a period of 14 days for each occurring episode. Their cold symptoms include fever, cough, runny nose, throat pain, headache, muscle pain, week feeling, hard breathing, retrosternal pain and lack of appetite. In addition, the subjects the duration of sickness by counting the start date of the beginning of any of the symptoms and the stop date of the last symptom. The duration of one URTI is defined as the number of days from the date of onset of any of the abovementioned symptoms to the date that all the symptoms are relieved.

The study product compliance was determined by counting the returned unconsumed sachets. The subjects were instructed to record each sachet that they took. Sufficient compliance was defined if 75 and 110 % of the sachets were consumed.

Outcome measures

The primary objective of the study is to evaluate the effects of 90-day beta-glucan consumption on the improvement of immunity in healthy individuals with having more than four times of common colds within the last 12 months. The incidence of URTIs was defined as the number of URTIs during the study period. The severity and duration of URTIs episodes, the incidence of medically confirmed adverse events and concomitant medications were assessed.

Processing of the blood and serum samples: blood samples of 12 normal individuals (5 males and 7 females) were obtained at Jinhua People's Hospital (Jinhua, Zhejiang Province, China). The serum samples were prepared using the standard method and the sera were stored at -80°C until analysis.

Serum Immune Biomarkers Measurement and Analysis: IFN γ, IL-2, and TNF-alpha/beta, IL-4, IL-5, IL-10 and IL-13, ((Th1: Th2 ratio (percentage of IFN γ and IL-4)), IL-12, IL 17, IL-6, IgE and CD44 at enrollment (baseline), 30-day, 60-day and 90-day visits.

In vitro cell proliferation assay (innate immunity): Peripheral Blood Mononuclear Cells (PBMCs) are cultured with or without con-canavalin A (Con A) for 72 h at 37C and label with [3H] thymidine, then assess DNA synthesis by measuring thymidine uptake at enrollment (baseline), 30-day, 60-day and 90-day visits.

Statistical analysis

This is a prospective, open label study in which all 12 subjects taking the same product. Data were summarized as mean ± standard deviation or median (the 25th percentile, the 75th percentile) for continuous variables, and frequency (percentage) for categorical variables. The summary URTIs symptoms were presented by age groups.

The comparison of the number and the duration of URTIs at the end of the study versus baseline were performed using paired t-test. The comparison of post-intervention blood biomarkers versus baseline were performed using paired t-test for each time point (day 30, 60 and 90). All statistical tests were performed at a significance level of 0.05.

Statistical analysis in this study was performed using SAS 9.3 statistical software (SAS Institute Inc., USA). All tests employed a 0.05 significance level.

Results

A total of 12 participants (18 to 80 years old) who sustained common cold (collectively URTIs) at least four times in the previous years were enrolled. All 12 subjects completed the trial. The gender ratio of completed participants was 5 males to 7 females (41.7%:58.3%), as shown in Table 1. The related symptoms of URTIs for all subjects are presented in Table 2.

Table 1. Baseline characteristics

Baseline characteristics	Summary statistics
Number of subjects	12
Males, %	5 (41.7%)
Females, %	7 (58.3%)
Age (years), mean (SD), median (q1, q3)	49.4 ± 17.2, 51.0 (38.0, 59.8)
18-30 years, %	2 (16.7%)
30-40 years, %	3 (25.0%)
50-60 years, %	4 (33.3%)
70-80 years, %	3 (25.0%)
Number of medical confirmed upper respiratory tract infections from the previous year during 3-month winter from December through March	1.33 ± 0.49, 1 (1, 2)
Average duration of upper respiratory tract from the previous year during 3-month winter from December through March	5.42 ± 1.16, 5 (4.75, 6.25)

Data are presented as mean±SD and median (Q1, Q3), or frequency (%).

SD: standard deviation; Q1: the 25th percentile; Q3: the 75th percentile.

Table 2. Symptoms of upper respiratory tract infections

Symptom	Overall (n=12)			18-30 years (n=2)			30-40 years (n=3)			50-60 years (n=4)			70-80 years (n=3)		
	Day 30	Day 60	Day 90	Day 30	Day 60	Day 90	Day 30	Day 60	Day 90	Day 30	Day 60	Day 90	Day 30	Day 60	Day 90
a. Fever	2 (16.7)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	2 (66.7)	0 (0.0)	0 (0.0)
b. Cough	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
c. Runny nose	6 (50.0)	2 (16.7)	0 (0.0)	1 (50.0)	0 (0.0)	0 (0.0)	1 (33.3)	0 (0.0)	0 (0.0)	2 (50.0)	1 (25.0)	0 (0.0)	2 (66.7)	1 (33.3)	0 (0.0)
d. Throat pain	4 (33.3)	2 (16.7)	0 (0.0)	1 (50.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	2 (50.0)	1 (25.0)	0 (0.0)	1 (33.3)	1 (33.3)	0 (0.0)
e. Headache	5 (41.7)	1 (8.3)	0 (0.0)	1 (50.0)	0 (0.0)	0 (0.0)	1 (33.3)	0 (0.0)	0 (0.0)	1 (25.0)	0 (0.0)	0 (0.0)	2 (66.7)	1 (33.3)	0 (0.0)
f. Muscle pain	1 (8.3)	1 (8.3)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (25.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (33.3)	0 (0.0)
g. Week feeling	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
h. Hard breathing	1 (8.3)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (33.3)	0 (0.0)	0 (0.0)
i. Retrosternal pain	2 (16.7)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	2 (66.7)	0 (0.0)	0 (0.0)
j. Lack of appetite	3 (25.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (33.3)	0 (0.0)	0 (0.0)	1 (25.0)	0 (0.0)	0 (0.0)	1 (33.3)	0 (0.0)	0 (0.0)

Table 3. Number and duration of upper respiratory tract infections and treatment

Outcomes	In the past year of baseline	During the study	Post-intervention vs. Baseline Difference (95% CI)	p-value
Number of medical confirmed upper respiratory tract infections	Per 3 months (December to March): 1.33±0,49, 1 (1, 2)	Per 3 months (December to March): 0.75±0.62, 1 (0, 1)	Per 3 months (December to March): -0.58 (-1.09, -0.08)	Per 3 months (December to March): 0.027
Average duration of sickness per URTI	Per 3 months (December to March): 5.42±1.16, 5 (4.75, 6.25)	Per 3 months (December to March): 2.71±2.22, 3 (0, 4.5)	Per 3 months (December to March): -2.71 (-3.61, -1.81)	Per 3 months (December to March): <0.0001
Total duration of sickness	Per 3 months (December to March): 7.42±3.78, 5.5 (5, 9)	Per 3 months (December to March): 2.92±2.31, 3.5 (0, 5)	Per 3 months (December to March): -4.50 (-6.39, -2.61)	Per 3 months (December to March): 0.0003

Data are presented as mean±SD and median (Q1, Q3).

SD: standard deviation; Q1: the 25th percentile; Q3: the 75th percentile.

The symptoms of URI for all subjects are recorded and summarized in Table 2. The durations of the time from start to stop of the common cold are recorded through the study but not for each single symptom.

The number and duration of upper respiratory tract infections and treatment for all subjects were recorded and summarized in Table 3.

The results presented in Table 3 showed that the incidence of the sickness for all subjects during the study are significantly less as compared with the same 3-month winter period from December through March from the previous year (p=0.027). The duration of the sickness per URTIs during the study are significantly less than from the previous year (p<0.0001). The total duration of sickness including URTIs are significantly less than the previous year (P=0.0003).

The results presented in Table 4 showed that statistically significant higher serum IFN-γ levels at day 30, day 60 and day 90 were observed as compared with the baseline (p<0.0001). Table 4 shows that statistically significant higher serum IL-2 levels at day 30 and day 60 (p<0.05) and day 90 (p>0.05 NOT statistical significant) were observed as compared with the baseline. Table 4 also

Table 4. Serum biomarkers

Biomarkers	Baseline	Day 30	Day 60	Day 90	Change compared to baseline (p-value)		
					Day 30 vs. Baseline	Day 60 vs. Baseline	Day 90 vs. Baseline
IgE (IU/ml)	19.54±3.32 18.95 (17.73, 21.35)	20.40±5.87 18.85 (17.20, 21.03)	20.85±3.08 19.80 (18.45, 22.35)	19.14±3.41 18.65 (16.10, 20.93)	0.563	0.074	0.676
IFN-γ (pg/ml)	1.16±0.06 1.17 (1.13, 1.21)	1.31±0.13 1.33 (1.23, 1.41)	1.29±0.11 1.32 (1.22, 1.37)	1.27±0.10 1.28 (1.21, 1.37)	<0.0001	0.0001	<0.0001
IL-4 (pg/ml)	1.46±0.16 1.45 (1.35, 1.55)	1.42±0.13 1.39 (1.31, 1.54)	1.45±0.15 1.42 (1.36, 1.53)	1.50±0.16 1.48 (1.39, 1.61)	0.357	0.904	0.188
IFN-γ: IL-4 ratio	0.80±0.09 0.81 (0.72, 0.85)	0.93±0.12 0.89 (0.84, 0.98)	0.89±0.11 0.91 (0.82, 0.97)	0.86±0.12 0.87 (0.78, 0.92)	0.005	0.010	0.043
IL-2 (pg/ml)	1.29±0.07 1.28 (1.25, 1.34)	1.71±0.65 1.39 (1.24, 1.97)	1.63±0.56 1.50 (1.23, 1.68)	1.40±0.21 1.35 (1.21, 1.58)	0.034	0.039	0.058
TNF-α (pg/ml)	1.92±0.72 1.72 (1.40, 2.19)	2.00±0.73 1.72 (1.49, 2.63)	1.93±0.73 1.75 (1.33, 2.31)	1.90±0.64 1.73 (1.45, 2.21)	0.204	0.789	0.489
IL-5 (pg/ml)	1.43±0.10 1.44 (1.35, 1.48)	1.51±0.26 1.43 (1.34, 1.60)	1.41±0.18 1.35 (1.30, 1.53)	1.41±0.17 1.35 (1.29, 1.49)	0.212	0.697	0.505
IL-6 (pg/ml)	4.41±0.48 4.62 (4.20, 4.70)	4.49±0.63 4.45 (4.15, 5.01)	4.42±0.52 4.63 (4.18, 4.71)	4.39±0.53 4.55 (4.25, 4.76)	0.379	0.903	0.397
IL-10 (pg/ml)	2.72±0.45 2.66 (2.36, 3.03)	2.91±0.66 2.71 (2.37, 3.36)	2.83±0.64 2.67 (2.30, 3.12)	2.70±0.46 2.67 (2.29, 2.97)	0.079	0.202	0.302
IL-12P70 (pg/ml)	2.10±0.32 2.17 (1.87, 2.34)	2.25±0.53 2.30 (1.82, 2.56)	2.12±0.44 2.26 (1.79, 2.38)	2.06±0.44 2.18 (1.72, 2.32)	0.123	0.588	0.449
IL-17A (pg/ml)	1.28±0.07 1.29 (1.24, 1.33)	1.20±0.15 1.24 (1.09, 1.33)	1.29±0.12 1.29 (1.20, 1.34)	1.32±0.10 1.31 (1.24, 1.39)	0.117	0.745	0.074
CD44 (pg/ml)	481.58±114.94 470.5 (403.5, 538)	486.42±117.67 459 (359.75, 559)	475.42±86.40 484.5 (395, 527.25)	477.00±101.80 474 (400.25, 536.25)	0.822	0.734	0.822
IL-13 (pg/ml)	56.55±17.29 62.25 (41.70, 70.30)	56.06±13.24 55.44 (51.43, 60.25)	58.21±13.31 (58.35 (49.95, 65.34)	57.59±11.12 56.43 (52.58, 61.88)	0.910	0.471	0.850
TNF-β (pg/ml)	1.53±1.01 1.35 (0.93, 1.80)	1.48±0.86 1.10 (0.90, 2.00)	1.58±0.88 1.55 (0.85, 2.33)	1.55±1.10 1.25 (0.80, 2.05)	0.744	0.696	0.859

Data are presented as mean±SD and median (Q1, Q3).

SD: standard deviation; Q1: the 25th percentile; Q3: the 75th percentile.

shows that statistically significant higher serum IFN-γ/IL-4 ratio at day 30, day 60 and day 90 were observed as compared with the baseline ($p<0.05$). However, no statistically significant changes were observed for other biomarkers at day 30, day 60 and day 90 as compared with the baseline.

The results presented in Table 5 showed that statistically significant higher IFN-γ levels at day 30, day 60 and day 90 were observed as compared with the baseline ($p<0.05$) from the in vitro PBMC cell proliferation assay. Table 5 also shows that statistically significant higher IFN- γ/IL-4 ratio at day 30 and day 60 ($p<0.05$) and day 90 ($p>0.05$ NOT statistical significant) were observed as compared with the baseline. However, no statistically significant changes were observed for other biomarkers at day 30, day 60 and day 90 as compared with the baseline (data from other biomarkers were not show).

Percentages of INF- γ and IL-4-producing CD4+ T cells (Th1:Th2 ratio) were determined by single-cell measurement of intracellular cytokines using flow cytometry as described (Openshaw P et.al. Heterogeneity of intracellular cytokine synthesis at the single-cell level in polarized T helper 1 and T helper 2 populations. (J Exp Med. 1995; 182: 1357–67).

Regarding adverse events, five events were observed for all subjects enrolled in the study: allergic rhinitis, frequent defecation, fracture, torticollis and cardiac murmurs. All adverse events were not related to the study products and none of these subjects were withdrawn from the study because of adverse events.

Conclusion & discussion

This is a Pilot, Open-label Study to evaluate on the effect of PureMune with Beta-Glucan on the improvement of immunity in healthy individuals during the flu season. A total of 100 people were screened and 24 of them were scheduled and visited the study site. A total of 12 subjects met the inclusion and exclusion criteria and enrolled into this study, and all 12 subjects finished this study with zero dropout.

The incidence of the sickness for all subjects during the study are significantly less as compared with the same 3-month winter period from December through March from the previous year ($p=0.027$). The duration of the sickness per URTI during the study are significantly less than from the previous year ($p<0.0001$). The total duration of sickness including URTI are significantly less than the previous year ($P=0.0003$).

Higher serum IFN-γ levels at day 30, day 60 and day 90 were observed as compared with the baseline ($p<0.0001$). Higher serum IL-2 levels at day 30 and day 60 ($p<0.05$) and day 90 ($p>0.05$ NOT statistical significant) were observed as compared with the baseline. Higher serum IFN-γ/IL-4 ratio at day 30, day 60 and day 90 were observed as compared with the baseline ($p<0.05$). However, there are no statistical significant changes that were observed for other serum biomarkers at day 30, day 60 and day 90 as compared with the baseline.

For the in vitro PBMC cell proliferation assay, higher IFN-γ levels at day 30, day 60 and day 90

Table 5. In Vitro PBMC Cell Proliferation Assay

Biomarkers	Baseline	Day 30	Day 60	Day 90	Change compared to baseline (p-value)		
					Day 30 vs. Baseline	Day 60 vs. Baseline	Day 90 vs. Baseline
IFN-γ (% CD4 + cells)	25.82±9.15 29.34 (20.06, 32.76)	31.20±10.69 32.51 (24.99, 38.43)	29.73±10.03 29.73 (22.89, 36.82)	28.21±10.04 30.86 (22.87, 33.44)	0.001	0.005	0.028
IL-4 (% CD4 + cells)	2.70±1.16 2.73 (1.65, 3.52)	2.50±1.20 2.34 (1.55, 3.50)	2.60±1.22 2.55 (1.44, 3.43)	2.80±1.23 2.77 (1.73, 3.91)	0.260	0.499	0.411
IFN-γ: IL-4 ratio	12.28±8.29 8.56 (6.96, 18.45)	15.62±9.91 12.39 (9.75, 19.76)	14.32±8.70 11.42 (8.90, 21.53)	12.61±7.60 10.35 (8.14, 20.82)	0.048	0.040	0.607
IL-2 (pg/ml)	1.58±0.14 1.60 (1.50, 1.66)	1.74±0.37 1.68 (1.43, 1.94)	0.66±0.23 1.76 (1.50, 1.82)	1.61±0.25 1.57 (1.46, 1.70)	0.071	0.092	0.555

Data are presented as mean±SD and median (Q1, Q3).

SD: standard deviation; Q1: the 25th percentile; Q3: the 75th percentile.

were observed as compared with the baseline ($p<0.05$). Higher IFN-γ/IL-4 ratio at day 30 and day 60 ($p<0.05$) and day 90 ($p>0.05$ NOT statistical significant) were observed as compared with the baseline. There are no statistical significant changes were observed for IL-2 and IL-4 at day 30, day 60 and day 90 as compared with the baseline.

Taken together, PureMune (30mg beta-glucan) consistently stimulate immune cells through the study (at 30, 60 and 90 days of intervention) for increasing the IFN-γ production both in vivo and in vitro. In addition, higher IFN-γ/IL-4 ratio at day 30, day 60 and day 90 for both in vivo and in vitro were observed as compared with the baseline ($p<0.05$). The higher ratio suggests that Th1:Th2 ratio was higher at day 30, 60 and 90 after receiving the beta-glucan than at the baseline because of the increasing IFN-γ (Th1 cells) and no percent change in IL-4 (Th2 cells) after receiving beta-glucan than at the baseline, which further suggest that intake of PureMune (beta-glucan) may be useful for prevention and treatment of infectious and allergic diseases induced by a weak Th1-type immune response.

These study results suggest that a daily intake of PureMune augments acquired immunity, especially Th1-related immune functions in healthy subjects. Augmentation of the Th1 response may be beneficial for individuals living in modern cities, because the better public hygiene and fewer infections in these societies may reduce the Th1 response, thereby increasing the risk of developing allergies.

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Phenotypic and genotypic testing (ESBL) and carbapenemases in multiresistant outpatient isolates

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Abstract

ESBL-producing strains were identified in 114 isolates, of which 60 isolates were *Escherichia coli* and 54 isolates of *Klebsiella pneumoniae*; Analysis of antibiogram results in isolates with molecularly proven ESBL and carbapenemase revealed resistance to AM-10 (ampicillin) in all 60/60 (100.00%) isolates of *E. coli* and in all 54/54 (100.00%) isolates *K. pneumoniae*; out of a total of 60 proven ESBL producing isolates of *E. coli*, the most proven is CTX-M G1 in 44 (73.3%), CTX-M G9 in 13 (21.7%), the variant CTX-M G9 + OXA-48 is proven is in 1 (1.7%), beta-lactamase SHV: 238S in 1 (1.7%) and SHV: 238S + 240K in 1 (1.7%); out of a total of 54 proven ESBL-producing isolates of *Klebsiella pneumoniae*, the most proven were CTX-M G1 in 53 (98.1%) and CTX-M G9 in 1 (1.9%); typing established the leading prevalence of CTX-M G1 beta-lactamases in isolates of outpatients in Sarajevo Canton; ESBL gene sequences were obtained for the first time in outpatients in Sarajevo Canton.

Key words: Carbapenemases, multiresistant outpatient isolates, *Escherichia coli*, *Klebsiella pneumoniae.*

Sažetak

ESBL – producirajući sojevi su utvrđeni kod 114 izolata, od toga 60 izolata je bilo *Escherichia coli* i 54 izolata *Klebsiella pneumoniae*; Analizom rezultata antibiograma kod izolata sa molekularno dokazanim ESBL i karbapenemazom, utvrđena je rezistencija na AM-10 (ampicilin) kod svih 60/60 (100,00%) izolata *E. coli* i kod svih 54/54 (100,00%) izolata *K. pneumoniae*; od ukupno dokazanih 60 ESBL producirajućih izolata *E. coli*, najviše dokazanih je CTX-M G1 kod 44 (73,3%), CTX-M G9 kod 13 (21,7%), varijanta CTX-M G9+OXA-48 dokazana je kod 1 (1,7%), beta-laktamaze SHV: 238S u 1 (1,7%) i SHV: 238S+240K u 1 (1,7%); od ukupno dokazanih 54 ESBL producirajućih izolata *Klebsiella pneumoniae*, najviše dokazanih je CTX-M G1 kod 53 (98,1%) i CTX-M G9 kod 1 (1,9%); tipizacijom je ustanovljena vodeća prevalencija CTX- M G1 beta-laktamaza kod izolata vanbolničkih pacijenata u Kantonu Sarajevo; prvi put su dobivene sekvence gena ESBL kod vanbolničkih pacijenata u Kantonu Sarajevo.

Ključne riječi: Karbapenemaze, multirezistentni vanbolnički izolati, *Escherichia coli*, *Klebsiella pneumoniae.*

Uvod

Većina bakterija posjeduje različite mehanizme kojima se postiže rezistencija na neki lijek. Jednom stečena, ona može da se prenosi na rezistentno potomstvo (1). Bakterije su kroz evoluciju do savršenstva razvile mehanizme za akumulaciju gena rezistencije na antibiotike. Geni locirani na hromozomu se prenose direktno na potomstvo (klonarno širenje), dok se geni locirani na plazmidima, transpozomima, integronima i bakteriofagima prenose horizontalno između bakterija istih ili različitih vrsta i rodova. Horizontalni transfer gena se odvija prenosom gena rezistencije na konjugabilne plazmide i transpozome, najčešće mehanizmima konjugacije, transpozicije i mjesto-specifične rekombinacije. Integroni nastaju kada se pojedinačne genske kasete integrišu mjesto-specifičnom rekombinacijom u jedinstvenu genetičku jedinicu, koja osim sistema za rekombinaciju obezbjeđuje i promotor za ekspresiju novougrađene DNK. Na ovaj način integroni formiraju operone gena za rezistenciju na antibiotike koji su dio transpozoma lociranih na konjugabilnim plazmidima. Kada se geni rezistencije

nađu na ovim elementima njihovo dalje širenje je samo pitanje vremena i selektivnog pritiska (2,3).

Iako prirodni antibiotici i mehanizmi rezistencije postoje i evoluiraju milionima godina u humanom okruženju, evolutivna bitka između antibiotika i gena rezistencije se drastično ubrzava uslijed velike koncentracije antimikrobnih agenasa. Selektivni pritisak koji nastaje upotrebom antibiotika favorizuje rezistentne klonove i smatra se osnovnim pokretačem nastanka i širenja rezistencije. Prekomjernom upotrebom antibiotika ne djelujemo samo selektivno na mehanizme rezistencije već i ubrzavamo evoluciju rezistencije. Rezistentne bakterije mogu da se šire ne samo unutar ograničenog područja, nego i dalje preko geografskih granica. Rasprostranjivanje rezistentnih patogena se može odvijati preko ljudi, životinja, životinjskih produkata ili kontaminacijom životne sredine (2,4).

Bakterije koje produciraju beta-laktamaze proširenog spektra (ESBL), osim u hospitaliziranih pacijenata, sve se češće izoliraju i u vanbolničkoj populaciji. Posljednjih godina veliki problem predstavljaju beta-laktamaze proširenog spektra (engl. *Extended-spectrum beta-lactamases*, ESBL) koje su široko rasprostranjene među enterobakterijama, predominantno u sojevima *Klebsiella pneumoniae* i *Escherichia coli* (5,6).

Nastanak rezistencije bakterija na više antimikrobnih lijekova istovremeno, postaje sve veći javnozdravstveni problem s obzirom na često sužen izbor efikasnih antibiotika ili čak odsustvo efikasnog lijeka za liječenje bakterijskih infekcija. Postoje dokazi da se kod gram-negativnih bakterija, naročito enterobakterija, geni rezistencije i pridruženi mobilni genetički elementi prisutni na plazmidima, često nalaze grupisani zajedno u velike multirezistentne regione (engl. *Multiresistance regions*). Veliki broj gena rezistencije u multirezistentnom regionu omogućava bakteriji brzo i istovremeno sticanje kombinacije gena rezistencije. Uslijed dejstva selektivnog pritiska, nastaju uspješne kombinacije koje se najčešće javljaju. Povezanost određenog gena rezistencije sa drugima može omogućiti njegovu koselekciju primjenom antibiotika iz drugih klasa (7).

Multirezistencija ili multipla rezistencija (engl. *Multidrug-resistant*-MDR) označava neosjetljivost ili smanjenu osjetljivost bakterija na najmanje po jedan antimikrobni lijek iz tri ili više klasa antibiotika.

ESBL su najučestalije izolirane u bakterija *Klebsiella pneumoniae* i *Escherichia coli* (8). ESBL su prvo izolirane u izolatima iz bolničkih infekcija, a danas i u vanbolničkim infekcijama (9). Antimikrobna rezistencija je prepoznata na globalnom nivou kao jedna od najvećih prijetnji za javno zdravlje ljudi. Naročito su značajne infekcije izazvane rezistentnim gram-negativnim bacilima, koji se sve češće bilježe širom svijeta (1).

Karbapenemi (imipenem, meropenem, ertapenem i doripenem) su beta-laktamski antibiotici širokog spektra tradicionalno smatrani prvom linijom odbrane protiv najtežih infekcija uzrokovanih otpornim sojevima gram-negativnih bacila.

Klasifikacija beta-laktamaza se zasniva ili na funkcionalnim karakteristikama enzima ili na njihovoj osnovnoj strukturi, odnosno molekularnim karakteristikama (10,11).

Početak molekularne klasifikacije predstavlja nalaz aminokiselinskih sekvenci četiri beta-laktamaze 1980. godine (12).

Beta-laktamaze proširenog spektra su enzimi koji se jako brzo razvijaju sa sposobnošću hidrolize i prouzrokovanja rezistencije na oksiminocefalosporine (cefotaksim, ceftazidim, ceftriakson, cefuroksim i cefepim), na monobaktame (aztreonam), ali ne i na cefamicine (cefoksitin i cefotetan) ili na karbapeneme (imipenem, meropenem i eritapenem) kod gram-negativnih bakterija, naročito kod *Escherichia coli* i *Klebsiella pneumoniae* (13,14,15). Rezistencija na beta-laktame kod enterobakterija najčešće nastaje enzimskom destrukcijom lijeka, dejstvom beta-laktamaza.

S obzirom na porijeklo, beta-laktamaze mogu biti hromozomske ili plazmidne (16). Hromozomske beta-laktamaze mogu biti konstitutivne ili inducibilne (16). Konstitutivne hromozomske beta-laktamaze se neprestano stvaraju, bez obzira na prisustvo beta-laktamskog antibiotika (17). Inducibilne hromozomske beta-laktamaze se stvaraju samo u slučaju kada je bakterija izložena djelovanju beta-laktamskog antibiotika (16). Plazmidne beta-laktamaze su kodirane prenosivim genetskim elementima-plazmidima i transpozomima. Plazmidne beta-laktamaze nisu specifične za vrstu kao hromozomske, već se mogu širiti među različitim vrstama i rodovima bakterija (18). Brojni rodovi gram-negativnih bakterija posjeduju hromozomski kodirane beta-lak-

tamaze (16). Nastajanje ovih beta-laktamaza se povezuje sa selektivnim pritiskom beta-laktamskih antibiotika koje luče neki mikroorganizmi prisutni u okolini (18). Paralelno sa uvođenjem novih antibiotika u liječenje infekcija, kao posljedica selektivnog pritiska i prevelike upotrebe, nastajale su nove vrste beta-laktamaza koje su uzrokovale otpornost (rezistenciju) bakterija na antibiotike. Posljedica primjene beta-laktamskih antibiotika bila je pojava i brzo širenje otpornosti putem plazmida i transpozoma (16).

Klasifikacija i nomenklatura beta-laktamaza je dugo bila problematična. Klasifikacije koje su do sada poznate su u osnovi funkcionalne (fenotipske) ili molekularne. Funkcionalne klasifikacije se zasnivaju na funkcionalnim karakteristikama beta-laktamaza, kao što su njihov hidrolitički spektar, osjetljivost na inhibitore, izoelektrična tačka ili molekularna težina. Molekularna klasifikacija je nastala na temelju određivanja slijeda aminokiselina (19).

Klasifikacija prema Bush-u dijeli beta-laktamaze u četiri skupine označene brojevima 1-4. Skupina 1 uključuje cefalosporinaze, koje ne inhibira klavulanska kiselina, a koje po molekularnoj klasifikaciji prema Ambleru spadaju u grupu C. Skupina 2 obuhvaća penicilinaze, cefalosporinaze ili beta-laktamaze, koje su inhibirane klavulanskom kiselinom, spadaju u molekularnu klasu A i D. Zbog rastućeg broja derivata TEM i SHV enzima ova skupina je podijeljena u dvije podskupine, 2a i 2b. Skupina 3 sadrži cink ili metalo-beta-laktamaze (karbapenemaze) koje odgovaraju molekularnoj klasi B. Skupina 4 uključuje penicilinaze koje nisu inhibirane klavulanskom kiselinom i nemaju još određenu molekularnu klasu. Molekularna klasifikacija beta-laktamaza prema Ambleru temelji se na slijedu nukleotida i aminokiselina u enzimima. Ambler je prvi 1980. godine klasificirao beta- laktamaze na osnovu molekularne strukture, u vrijeme kada su bile poznate samo četiri sekvence aminokiselina beta-laktamaza (20). Prema ovoj klasifikaciji beta-laktamaze su podijeljene u četiri grupe označene slovima A-D.

Ispitanici i metode istraživanja

Studija je realizovana kao prospektivna, u vremenskom periodu 2017/2019. godina u Mikrobiološkom laboratoriju Zavoda za javno zdravstvo Kantona Sarajevo i u Službi za spremljanje občutljivosti bakterij in gliv, Laboratorij za molekularno bakteriologijo in mikologijo, Inštitut za mikrobiologijo in imunologijo, Medicinska fakulteta Univerza v Ljubljani.

Istraživanjem su obuhvaćeni multirezistentni izolati gram-negativnih bakterija, uzročnika infekcija: *Escherichia coli* i *Klebsiella pneumoniae* izolovanih iz urina i drugih bioloških uzoraka vanbolničkih pacijenata u Kantonu Sarajevo. U ispitivanje rezistencije uključivao se samo prvi izolat određene bakterijske vrste izoliran u istog pacijenta. Istraživanjem se sprovelo dokazivanje beta-laktamaza proširenog spektra (ESBL) i karbapenemaza na uzorku od 114 multirezistentnih izolata.

Svim pacijentima su uzimani biološki uzorci u okviru rutinske, medicinski indicirane bakterioloških analize. Biološki materijali su inokulirani na odgovarajuće hranljive podloge i propisno inkubirani. Gram- negativni izolati su se identificirali standardnim mikrobiološkim metodama.

Lančana reakcija polimeraze (PCR) je najčešće korištena metoda za detekciju i identifikaciju beta-laktamaza u kojoj se koriste oligonukleotidni začetnici („primeri“) specifični za gene određenih beta-laktamaza. Za *E.coli* i *K.pneumoniae* su se određivali CTX-M PCR (multiplex PCR) i prisutnost TEM i SHV ESBL enzima (PCR+sekvenciranje). Primijenio se klasični PCR: CTX-M PCR (123).

Nakon uočene smanjene osjetljivosti sojeva na karbapeneme, potrebno je provesti potvrdna testiranja za produkciju karbapenemaza. Postoji nekoliko mogućih metoda od kojih je novim EUCAST-ovim smjernicama preporučena fenotipska metoda upotrebom kombiniranih diskova (engl. *combination disk test*, CTD).

Carba NP test (engl. *Carbapenemase Nordmann-Poirel*) je kolorimetrijski test zasnovan na hidrolizi karbapenema od karbapenemaza producirajućeg soja, te posljedično produkciji kiseline, padu pH i promjeni boje indikatora. CIM test (*carbapenem inactivation method* CIM) se zasniva na inaktivaciji imipenema u antibiotičnom disku.

Najpouzdanija metoda detekcije karbapenemaza je amplifikacija gena koji ih kodiraju lančanom reakcijom polimeraze (engl. *polymerase chain reaction*, PCR). Rutinski se utvrđuju KPC, VIM, IMP, NDM i OXA-48. (Kit Light Mix Modular Carbapenemases).

Rezultati istraživanja

U Laboratoriju za molekularnu bakteriologiju i mikologiju Instituta za mikrobiologiju i imunologiju Medicinskog fakulteta Univerziteta u Ljubljani, testirano je 114 ESBL- producirajućih izolata PCR metodom. Od toga 60 (47%) je bilo *Escherichia coli* i 54 (53%) *Klebsiella pneumoniae*.

Tabela 1. Prisustvo karbapenemaze i ESBL

	Escherichia coli		***Klebsiella pneumonia***	
	N	**%**	**N**	**%**
ESBL	59	98.3%	49	90.7%
ESBL CPE	1	1.7%	0	0.0%
ESBL CRE	0	0.0%	5	9.3%
Ukupno ESBL	60	100.0%	54	100.0%
Bez	59	98.3%	49	90.7%
CPE	1	1.7%	0	0.0%
CRE	0	0.0%	5	9.3%

Od ukupno 114 ESBL-producirajućih izolata testiranih PCR metodom, 60 izolata je detektovana *Escherichia coli* i 54 izolata *Klebsiella pneumoniae*.

Od ukupno 60 PCR testiranih izolata *E. coli*, produkcija enzima karbapenemaza utvrđena je kod 1 (1,7%) izolata *E. coli*. Utvrđeno je 59 (98,3%) ESBL-producirajućih sojeva *E. coli*, dok CRE (carbapenem rezistentni) soj nije utvrđen kod izolata *E. coli*.

Od ukupno 54 PCR testiranih izolata *K. pneumoniae*, produkcija enzima karbapenemaza nije utvrđena kod izolata *K.pneumoniae*. Utvrđeno je 49 (90,7%) ESBL-producirajućih sojeva *K. pneumoniae*, a ESBL CRE (carbapenem rezistentni) soj je utvrđen kod 5 (9,3%) izolata *K. pneumoniae*.

Molekularnim metodama, u našem istraživanju je izvršena detekcija ESBL gena i utvrđeno je postojanje različitih gena u vanbolničkim izolatima *Escherichiae coli* i *Klebsiella pneumoniae*. Kod izolata *E. coli* : 44 su bili blaCTX-M G1, 13 blaCTX-M G9, 1 blaSHV; 238S i 1 blaSHV; 238S+240K. Kod izolata *K.pneumoniaei* : 48 su bili blaCTX-M G1 i 1 blaCTX-M G9.

Od ukupno 60 ESBL producirajućih sojeva *Escherichiae coli*, kod 44 izolata je utvrđen gen blaCTX-M G1, kod 13 izolata gen blaCTX-M G9, kod 1 izolata ESBL-CPE utvrđen je gen blaCTX-M G9; blaOXA-48, kod 1 izolata utvrđen je gen blaSHV; 238S i kod 1 izolata gen blaSHV; 238S+240K.

Od ukupno 54 ESBL producirajućih sojeva *Klebsiella pneumoniae*, 49 su bili ESBL ,a 5 ESBL-CRE. Od ukupno 49 ESBL producirajućih sojeva, kod 48 je utvrđen gen blaCTX-M G1 i kod 1 blaCTX-M G9.

Gen blaCTX-M G1 utvrđen je kod 44 ESBL sojeva *E.coli*. Gen blaCTX-M G9 utvrđen je kod 13 ESBL sojeva *E.coli*. Gen blaCTX-M G9; blaOXA-48 utvrđen je kod 1 ESBL-CPE soj *E.coli.* Gen blaSHV; 238S utvrđen je u 1 ESBL soju *E.coli*. Gen blaSHV; 238S+240K utvrđen je u 1 ESBL soju *E.coli*.

Gen blaCTX-M G1 utvrđen je kod 48 ESBL sojeva *Klebsiellae pneumoniae* i 5 ESBL-CRE sojeva *Klebsiella pneumoniae*. Gen blaCTX-M G9 utvrđen je kod 1 ESBL soja *Klebsiella pneumoniae*.

Kod ESBL producirajućih sojeva *E.coli*, gen blaCTX-M G1 potvrđen je u 44 (73,3%), gen blaCTX-M G9 u 13 (21,7%), gen blaCTX-M G9; blaOXA-48 potvrđen je u 1 (1,7%) ESBL-CPE, gen blaSHV; 238S potvrđen je u 1 (1,7%) ESBL *E.coli* i gen blaSHV; 238S+240K u 1 (1,7%) ESBL *E.coli*.

Kod ESBL producirajućih sojeva *Klebsiella pneumoniae*, gen blaCTX-M G1 potvrđen je kod 48 (88,9%) ESBL i kod 5 (9,3%) sojeva *K.pneumoniae*. Gen blaCTX-M G9 potvrđen je kod 1 (1,9%) ESBL producirajućeg soja *Klebsiella pneumoniae*.

Kombinacija gena blaCTX-M G9; blaOXA-48 utvrđena je kod 1 (1,7%) ESBL-CPE soja *E.coli*. Kombinacija gena blaSHV; 238S utvrđena je u 1 (1,7%) ESBL producirajućem soju *E.coli*. Kombinacija gena blaSHV; 238S+240K utvrđena je u 1 (1,7%) ESBL producirajućem soju *E.coli* .

Od ukupno potvrđenih beta-laktamaza kod 60 ESBL producirajućih sojeva *E.coli*, 44 (73,3%) su CTX-M G1, 13 (21,7%) CTX-M G9, 1 (1,7%) su CTX-M G9; OXA-48, 1 (1,7%) SHV; 238S i 1 (1,7%) SHV; 238S+240K.

Od ukupno potvrđenih beta-laktamaza kod 54 ESBL producirajućih sojeva *K. pneumoniae*, 53 (98,1%) su CTX-M G1 i 1 (1,9%) CTX-M G9.

Tabela 2. Prisustvo beta-laktamaza u odnosu na analiziranu bakteriju

	Escherichia coli		***Klebsiella pneumonia***	
Beta-laktamaze	N	%	N	%
CTX-M G1	44	73,3%	53	98,1%
CTX-M G9	13	21,7%	1	1,9%
CTX-M G9; OXA-48	1	1,7%	0	0,0%
SHV: 238S	1	1,7%	0	0,0%
SHV: 238S+240K	1	1,7%	0	0,0%

Kod ESBL- producirajućih izolata *Escherichiae coli* dokazano je prisustvo CTX-M G1 kod 44 (73,3%), CTX-M G9 kod 13 (21,7%), koprodukcija CTX-M G9 i OXA-48 kod 1(1,7%), SHV: 238S kod 1 (1,7%) i SHV: 238S+240K kod 1 (1,7%).

Kod ESBL-producirajućih izolata *Klebsiellae pneumoniae* dokazano je prisustvo CTX-M G1 kod 53 (98,1%), CTX-M G9 kod 1 (1,9%). Kod ovih izolata nije utvrđeno postojanje CTX-M G9 u koprodukciji sa OXA-48, kao ni SHV: 238S, te SHV.238S+240K, što je potvrđeno kod testiranih izolata *Escherichiae coli*.

Diskusija

Multirezistentni sojevi mogu vertikalnim transferom da prenose determinante rezistencije i omoguće širenje soja i njegovu povećanu zastupljenost. Također, ovi sojevi mogu da postanu donori i da horizontalno prenesu determinante rezistencije na druge sojeve, vrste i rodove (21). Dodatni problem predstavljaju multirezistentni regioni koji imaju pluripotentni potencijal rezistencije, tako da upotreba jednog antibiotika dovodi do rezistencije na mnoge druge. Proučavanjem različitih mehanizama rezistencije naročito među bakterijama nađenim u prirodi, stičemo znanja o novim mogućim mehanizmima.

Geni rezistencije nađeni u prirodi mogu da se prenesu na enterobakterije značajne za medicinu, ali i obrnuto, sa humanih patogena geni rezistencije mogu putem otpadnih voda, vodotokova, životinja ili biljaka da dospiju u prirodu. Registrovanjem i praćenjem multirezistentnih sojeva poboljšava se nadzor i omogućava ciljana primjena neophodnih mjera za suzbijanje epidemija. Bolja kontrola infekcija adekvatnom higijenom ruku, korištenjem zaštitnih sredstava naročito prilikom njege pacijenata kolonizovanih ili inficiranih rezistentnim sojevima, značajno doprinosi sprječavanju širenja multirezistentnih sojeva enterobakterija u bolnicama. U nedostatku novih antibiotskih agenasa koji bi bili razvijeni u bliskoj budućnosti, djeluje da se bližimo kraju antibiotske ere. Sa ciljem naglašavanja ozbiljnosti situacije i poređenjem sa klasičnim svjetskim pandemijama, epidemija rezistencije Gram-negativnih bakterija je nazvana „Crvena kuga“ (22).

Naše istraživanje je obuhvatilo 114 multirezistentnih vanbolničkih izolata gram-negativnih bakterija, testiranih u Mikrobiološkom laboratoriju Zavoda za javno zdravstvo Kantona Sarajevo i Laboratoriju za molekularnu bakteriologiju i mikologiju Instituta za mikrobiologiju i imunologiju Medicinskog fakulteta Univerziteta u Ljubljani.

U našem istraživanju utvrđeno je postojanje različitih gena u vanbolničkim izolatima *Escherichiae coli* i *Klebsiella pneumoniae*. Kod izolata *E. coli* : 44 su bili blaCTX-M G1, 13 blaCTX-M G9, 1 blaSHV; 238S i 1 blaSHV; 238S+240K. Kod izolata *K.pneumoniaei* : 48 su bili blaCTX-M G1 i 1 blaCTX-M G9.

Od ukupno 60 ESBL producirajućih sojeva *Escherichiae coli*, kod 44 izolata je utvrđen gen blaCTX-M G1, kod 13 izolata gen blaCTX-M G9, kod 1 izolata ESBL-CPE utvrđen je gen blaCTX-M G9; blaOXA-48, kod 1 izolata utvrđen je gen blaSHV; 238S i kod 1 izolata gen blaSHV; 238S+240K.

Od ukupno 54 ESBL producirajućih sojeva *Klebsiella pneumoniae*, 49 su bili ESBL ,a 5 ESBL-CRE. Od ukupno 49 ESBL producirajućih sojeva, kod 48 je utvrđen gen blaCTX-M G1 i kod 1 blaCTX-M G9.

Gen blaCTX-M G1 utvrđen je kod 44 ESBL sojeva *E.coli*. Gen blaCTX-M G9 utvrđen je kod 13 ESBL sojeva *E.coli*. Gen blaCTX-M G9; blaOXA-48 utvrđen je kod 1 ESBL-CPE soj *E.coli.* Gen blaSHV; 238S utvrđen je u 1 ESBL soju *E.coli*. Gen blaSHV; 238S+240K utvrđen je u 1 ESBL soju *E.coli*.

Gen blaCTX-M G1 utvrđen je kod 48 ESBL sojeva *Klebsiellae pneumoniae* i 5 ESBL-CRE sojeva *Klebsiella pneumoniae*. Gen blaCTX-M G9 utvrđen je kod 1 ESBL soja *Klebsiella pneumoniae*.

Kod ESBL producirajućih sojeva *E.coli*, gen blaCTX-M G1 potvrđen je u 44 (73,3%), gen blaCTX-M G9 u 13 (21,7%), gen blaCTX-M G9; blaOXA-48 potvrđen je u 1 (1,7%) ESBL-CPE,

gen blaSHV; 238S potvrđen je u 1 (1,7%) ESBL *E.coli* i gen blaSHV; 238S+240K u 1 (1,7%) ESBL *E.coli*.

Kod ESBL producirajućih sojeva *Klebsiella pneumoniae*, gen blaCTX-M G1 potvrđen je kod 48 (88,9%) ESBL i kod 5 (9,3%) sojeva *K.pneumoniae*. Gen blaCTX-M G9 potvrđen je kod 1 (1,9%) ESBL producirajućeg soja *Klebsiella pneumoniae*.

Kombinacija gena blaCTX-M G9; blaOXA-48 utvrđena je kod 1 (1,7%) ESBL-CPE soja *E.coli*. Kombinacija gena blaSHV; 238S utvrđena je u 1 (1,7%) ESBL producirajućem soju *E.coli*. Kombinacija gena blaSHV; 238S+240K utvrđena je u 1 (1,7%) ESBL producirajućem soju *E.coli*.

Od ukupno potvrđenih beta-laktamaza kod 60 ESBL producirajućih sojeva *E.coli*, 44 (73,3%) su CTX-M G1, 13 (21,7%) CTX-M G9, 1 (1,7%) su CTX-M G9; OXA-48, 1 (1,7%) SHV; 238S i 1 (1,7%) SHV; 238S+240K.

Od ukupno potvrđenih beta-laktamaza kod 54 ESBL producirajućih sojeva *K. pneumoniae*, 53 (98,1%) su CTX-M G1 i 1 (1,9%) CTX-M G9.

Objavljena su mnoga istraživanja u kojima je molekularna tipizacija korištena u proučavanju epidemiologije bolničkih infekcija uzrokovanih sa ESBL producirajućim izolatima (23). Prije razvoja metoda molekularne biologije, u procjeni genske povezanosti bolničkih mikroorganizama, korištene su fenotipske metode. Te metode uključuju biotipizaciju i procjenu osjetljivosti na antimikrobne lijekove (24,25). Većina istraživača danas koristi molekularne metode u određivanju genetičke srodnosti ESBL-producirajućih izolata.

U posljednjih nekoliko godina došlo je do širenja infekcija uzrokovanih ESBL sojevima i u vanbolničkoj sredini (26,27,28). ESBL izolati iz vanbolničke sredine su prvi put zabilježeni 1998. godine u Irskoj, kod E.coli otporne na nalidiksičnu kiselinu, a koja je bila izolirana iz urina starijih pacijenata; tip enzima nije bio određen; pacijent nije bio ranije hospitaliziran, ali je primao višestruke kurseve antibiotika (29).

U epidemiologiji ESBL treba uzeti u obzir više različitih nivoa, kao što je individualni pristup svakom pojedinom pacijentu, vrstu medicinske institucije i geografsko područje (30).

Među gram-negativnim bakterijama koje ne pripadaju enterobakterijama uzročnici koji proizvode ESBL su mnogo rjeđi, među njima najvažniji su izolati Pseudomonas aeruginosa. Ovi mikroorganizmi su domaćini OXA tipa beta-laktamaza, međutim, oni proizvode i TEM, SHV i PER ESBL koje su otkrivene u nekim državama svijeta (30).

Postoje mnogobrojna izvješća o ESBL iz cijelog svijeta. Prevalencija ESBLs se kreće od 10-40% kod K.pneumoniae i oko 4% u E.coli (31). Prevalencija ESBL s u Europi je veća u odnosu na SAD, ali niža u odnosu na azijske i sjeverno američke države (32). U periodu od 2009. do 2011. godine prevalencija zastupljenosti ESBL s kod E.coli u Pakistanu, Iraku, Iranu i Indiji se kretala od 43-96%, a kod Klebsiella spp. 60% (33).

U istraživanju iz 2016. god., fenotipski test za dokazivanje produkcije beta-laktamaza proširenog spektra kod izolata sa smanjenom osjetljivošću na bar jedan karbapenem je bio pozitivan kod polovine ispitivanih izolata. Fenotipski test za dokazivanje karbapenemaza kod izolata sa smanjenom osjetljivošću na bar jedan karbapenem bio je pozitivan kod nešto više od trećine izolata (36,3%). Kod svih izolata osjetljivih na karbapeneme, fenotipski test za utvrđivanje prisustva karbapenemaza je bio negativan. Utvrđena je statistički značajna razlika među izolatima sa smanjenom osjetljivošću na bar jedan karbapenem i izolata osjetljivih na karbapeneme u odnosu na rezultat fenotipskog testa. Kod 179 izolata sa smanjenom osjetljivošću na bar jedan karbapenem, geni koji kodiraju karbapenemaze (blaKPC,blaNDM,blaVIM,blaIMP,blaOXA-48 like) nađeni su kod 79 (44,1%) izolata. Kod izolata osjetljivih na karbapeneme nije nađen ni jedan gen koji kodira karbapenemaze. Utvrđena je statistički značajna razlika među izolatima sa smanjenom osjetljivošću na bar jedan karbapenem i izolata osjetljivih na karbapeneme u odnosu na prisustvo gena koji kodiraju karbapenemaze. Korištenim fenotipskim testom za dokazivanje karbapenemaza moglo je da se dokaže prisustvo metalo-beta laktamaza klase B, karbapenemaza iz klasa A i da se eliminiše prisustvo AmpC tipa cefalosporinaza. Od 65 izolata sa pozitivnim fenotipskim testom za dokazivanje karbapenemaza, 63 su ukazivala na prisustvo metalo-beta laktamaza, a 2 su ukazivala na prisustvo karbapenemaza iz grupe A. Geni koji kodiraju karbapenemaze su nađeni kod 60 izolata sa pozitivnim fenotipskim testom, dok kod 5 izolata nisu detektovani geni koji ko-

diraju karbapenemaze. Senzitivnost fenotipskog testa za dokazivanje karbapenemaza klase A i B iznosila je 100,0%, specifičnost 96,6%, a ukupna tačnost testa bila je 97,6%). Visoka senzitivnost od 100% i specifičnost od 98,8% fenotipskog testa za dokazivanje karbapenemaza klase A i B potvrđena je od strane drugih autora (34,35,36).

Prvi objavljeni radovi o infekcijama izazvanim izolatima koji produkuju NDM karbapenemaze odnosili su se na osobe koje su zdravstvenu njegu primali u Indiji, iako precizno geografsko porijeklo i tačno vrijeme pojave blaNDM gena nisu poznati (221). Enzim NDM je prvi put izolovan u januaru 2008. godine kod muškarca indijskog porijekla koji je živio u Švedskoj i tokom više godina putovao u Indiju, gdje je bio hospitalizovan. Dan nakon prijema u bolnicu u Švedskoj izolovan je multirezistentan soj Klebsiella pneumoniae rezistentan na karbapeneme. U martu 2008. godine iz uzorka stolice istog pacijenta izolovana je Escherichia coli rezistentna na karbapeneme. Budući da PCR-om nisu nađeni do tada poznati geni, kloniranjem i sekvenciranjem utvrđeno je da se radi o novoj metalo-beta-laktamaza nazvanoj NDM-1 (37,38). Naredne godine NDM-1 enzim je nađen kod 29 pacijenata u Ujedinjenom Kraljevstvu. Iste godine gen blaNDM je identifikovan kod 143 pacijenta sa brojnih mjesta na indijskom potkontinentu (39). Indija i Pakistan se smatraju endemskim područjem za bakterije koje produkuju NDM. Prenos i širenje NDM karbapenemaza po cijelom svijetu se desio zahvaljujući putovanjima, „medicinskom turizmu“ i sposobnosti genetičkih elemenata da se prenose među bakterijama (40).

Prava prevalencija OXA-48 enzima je nedovoljno poznata, budući da enzimi posjeduju varijabilnu aktivnost u odnosu na karbapeneme i teško se detektuju fenotipskim metodama (41).

Kod više od 85% izolata sa dokazanim genima koji produkuju karbapenemaze dokazano je istovremeno prisustvo i blaCTX-M gena koji kodira beta-laktamaze proširenog spektra. Kod svih izolata koji pokazuju smanjenu osjetljivost na karbapeneme, a ne produkuju karbapenemaze, dokazano je prisustvo bla CTX-M gena. Rezistencija na karbapeneme, pored prisustva karbapenemaza, može nastati uslijed gubitka porina u kombinaciji sa produkcijom beta-laktamaza proširenog spektra ili AmpC beta-laktamaza. Naročito prisustvo CTX-M enzima u kombinaciji sa gubitkom porina može uzrokovati rezistenciju na ertapenem (42). Ertapenem se smatra indikatorom visoke senzitivnosti za dokazivanje produkcije karbapenemaza kod bakterija (43). Međutim, ertapenem pokazuje nisku specifičnost za detekciju karbapenemaza u odnosu na meropenem i imipenem (44).

Zaključci

Od ukupno dokazanih 60 ESBL multirezistentnih izolata *E. coli*, najviše dokazanih je CTX-M G1 kod 44 (73,3%), CTX-M G9 kod 13 (21,7%), varijanta CTX-M G9+OXA-48 dokazana je kod 1 (1,7%), beta-laktamaze SHV: 238S u 1 (1,7%) i SHV: 238S+240K u 1 (1,7%).

Od ukupno dokazanih 54 ESBL multirezistentnih izolata *Klebsiella pneumoniae*, najviše dokazanih je CTX-M G1 kod 53 (98,1%) i CTX-M G9 kod 1 (1,9%).

Tipizacijom je ustanovljena vodeća zastupljenost CTX- M G1 beta-laktamaza kod izolata vanbolničkih pacijenata u Kantonu Sarajevo.

Prvi put su detektovani bla-geni iz ESBL-producirajućih sojeva kod vanbolničkih pacijenata u Kantonu Sarajevo.

Rezultati istraživanja predstavljaju prvi izvještaj prisustva OXA-48 karbapenemaza kod izolata vanbolničkih pacijenata u Kantonu Sarajevo.

Prvi put je detektovan soj *E. coli* sa koprodukcijom CTX-MG9 i OXA-48 karbapenemaza kod izolata vanbolničkih pacijenata u Kantonu Sarajevo.

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Efficiency of the high bioavailable magnesium salt ATAMg® on premenstrual syndrome

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Abstract

Premenstrual syndrome (PMS) has a wide variety of signs and symptoms, including mood swings, tender breasts, food cravings, fatigue, irritability and depression. It's estimated that as many as 1 of every 2 menstruating women have experienced some form of premenstrual syndrome. Symptoms tend to recur in a predictable pattern. But the physical and emotional changes women experience with premenstrual syndrome may vary from just slightly noticeable all the way to intense. Treatments and lifestyle adjustments, like a supplementation of a specific source of magnesium can help to reduce or manage the signs and symptoms of premenstrual syndrome. The positive clinical results obtained with ATAMg® are very encouraging to offer an alternative to the medicinal drugs to help women who suffer from premenstrual syndrome and who prefer to use a natural solution during their menstrual cycles.

Key words: *Efficacy of ATAMg®, Premenstrual syndrome (PMS), Clinical study, Subjects under medical control, Menstrual cycles, Recording of possible adverse events.*

Introduction

It is widely recognized that many women experience cyclical changes in somatic, behavioral and affective symptoms in relation to the menstrual cycle[1]. The most consistently reported finding is of a premenstrual increase in negative symptoms; hence the frequently employed nomenclature, premenstrual syndrome (PMS). Despite considerable study, the incidence and etiology of PMS remains unclear. Part of this confusion reflects methodological differences in the study and conceptualization of the disorder.

More than 150 diverse symptoms have been reported to occur, or become exacerbated, premenstrually[2-4]. Changes in these symptoms across the menstrual cycle are typically assessed using pen and paper questionnaires. The most widely recognized and used questionnaire is the Moos Menstrual Distress Questionnaire. Ross and al. wrote an- other scientific paper which deals with factor structure of the Modified Moos Menstrual Distress Questionnaire: assessment of prospectively reported follicular, menstrual and premenstrual symptomatology[5].

Magnesium deficiency is considered as a contribution factor to some symptoms of PMS. Several studies have re- ported a lower intracellular magnesium concentration in women with PMS[6]. The mechanism by which magnesium deficiency induces PMS symptoms has not been fully elucidated, but several hypotheses have been proposed.

This magnesium depletion may affect the vascular system, synaptic transmission, and excitation-secretion coupling, and thus may produce some of the well-known symptoms of PMS[7]. In addition, some PMS symptoms have been found to share common characteristics associated with magnesium deficiency.

In the absence of an adequate amount of magnesium, NMDA receptors are hyperactivated, resulting in an overactivation of brain function, which is involved in several neurological disorders observed in PMS: migraine, stress, anxiety, etc[8].

Magnesium depletion in cerebrovascular smooth muscle cells can also lead to vasospasm, which may be involved in migraines[9]. In addition, hormonal changes also lead to inflammation, particularly in the central nervous system[10]. Inflammation leads to inappropriate activation of glutamate receptors, which are known to play a role in pain transmission[11].

Therefore, one of the proposed approaches to PMS prevention is magnesium supplementation. The double objective of this clinical study is to

highlight the efficiency of ATA Mg® on Premenstrual Syndrome (PMS) in an open intra-individual study; each female subject is her own control; and then, to record some eventual adverse events after the ATA Mg® oral daily intake. The study was conducted in 2020, over a period of 3 menstrual cycles.

Methods

Population recruitment

Selection

- Specific criteria

19 women from 18 to 45 years old (average: 33 years old), having regular menstrual cycles, every 24 to 31 days and lasting at least 3 days were selected.

All subjects present symptoms of Premenstrual Syndrome with one of the symptoms requiring treatment according to the patient (declarative + preselection questionnaire for confirmation of inclusion by the clinician).

All women were also chosen with inadequate magnesium intake from food.

- General criteria

All women were healthy subjects, having given her free in- formed, written consent and willing to adhere to the protocol and study procedures.

- Exclusion criteria

- Subjects presenting symptoms of COVID–19 (moderate fever, dry cough, and other symptoms as described by the World Health Organization);
- Subjects with a temperature higher than 37.5°C;
- Subject having been tested positive for COVID-19 and without a medical certificate from the Government;
- Pre-menopausal or post-menopausal subjects;
- Subjects on antibiotics: Quinolone, tetracycline, etc.;
- Subjects on IPP: Omeprazole®;
- Subjects in renal failure;
- Subjects with hypotension;
- Subjects having any gynecological condition liable to interfere with the assessment of the efficacy of the study product;
- Subjects with metabolic disease;
- Subjects with known psychiatric pathology;
- Subjects who smoke;
- Subjects with a history of alcohol abuse or illicit substances;
- Subject not using the same oral contraception method for more than one year;
- For women: pregnant or nursing woman or woman planning to get pregnant during the study;
- Use of topical or systemic treatment during the previous weeks liable to interfere with the assessment of the efficacy of the study product;
- Subject having undergone a surgery under general anesthesia within the previous month
- Consumption of other dietary supplements during the study;
- Subject planning to change their lifestyle and eating habits;
- Subject enrolled in another clinical trial during the study period.

- Oral administration

ATA Mg® capsules was administered at the rate of 770 mg daily; 1 capsule of 385 mg twice daily (one in the morning, the other one, at night).

Study requirements and constraints

All patients had to respect dates and hours of evaluation visits, followed the conditions of use of the investigational products at home and completed the daily log and brought it back with the investigational products at the end of the study.

They must not take other types of food supplements, allowed the use of the study product by another person than herself and changed their lifestyles and eating habits.

Protocol deviations

A protocol deviation can be defined as any non-adherence to the final protocol, including:

- wrong inclusion (inclusion criteria or non-inclusion criteria not fulfilled);

- start of a prohibited concomitant treatment;
- non-adherence of the subjects to the study schedule (missed or postponed visit);
- missing data for one or several evaluations criteria;
- low compliance of the subject to the study product(s) application;
- premature study end or untraceable subject;
- no respect of the constraints envisaged by the protocol.

Deviations to the protocol were classified as:

- **minor** if they don't impact the rights, safety or well-being of the subjects. They do not increase the risk for the subject and/or do not have a significant effect on the integrity of the data collected,
- **major** (or protocol violations) if they affect the rights, safety or well-being of participants. They increase the risk for the subject and/or have a significant effect on the integrity of the study data,
- **critical**: any protocol violations as mentioned above necessarily requiring the suspension or the termination of the study.

The protocol non-adherences of Subject #70 and Subject #88 induced their exclusion from the analysis (major non-adherence) and their data were not kept in the analysis.

Among the 21 women included at the beginning of the clinical study, finally 19 were definitely recruited. Subjects' characteristics are presented in the Table 1, here-below.

Study process

Concomitant treatments

None of the concomitant medications started after the beginning of the study invalidated the data obtained for the subjects in question. The concomitant medications are shown in Table 2.

Table 1. Women' characteristics

Subject#	Last name	First name	Age	Having PMS symptoms	Duration of menstrual cycle	Duration of menses (day/s)	Last menstrual period	Comments
3	FA	J	26	Yes	28	4	30/Aug/20	None
6	MA	T	23	Yes	28	4	30/Aug/20	None
10	PA	T	26	Yes	31	4	31/Aug/20	None
13	RA	M	45	Yes	24	5	8/Sep/20	None
16	CU	M	33	Yes	28	3	8/Sep/20	None
23	LE	M	30	Yes	30	4	14/Sep/20	None
27	AG	M	29	Yes	25	5	20/Sep/20	None
34	CA	K	41	Yes	28	3	20/Sep/20	None
38	LI	M	27	Yes	26	3	20/Sep/20	None
48	AN	L	39	Yes	30	4	30/Sep/20	None
50	HE	B	35	Yes	28	4	29/Sep/20	None
52	BA	I	29	Yes	28	4	28/Sep/20	None
56	BI	Y	28	Yes	25	4	28/Sep/20	None
66	BE	D	37	Yes	28	4	3/Oct/20	None
(70)*	(AG)*	(C)*	(25)*	(Yes)*	(28)*	(4)*	(13/Oct/20)*	(Protocol deviation)*
76	BA	J	28	Yes	29	4	14/Oct/20	None
79	MA	C	34	Yes	28	4	14/Oct/20	None
81	RA	B	26	Yes	28	3	20/Oct/20	None
83	BA	I	45	Yes	29	5	18/Oct/20	None
85	MA	S	42	Yes	28	3	18/Oct/20	None
(88)*	(SA)*	(A)*	(22)*	(Yes)*	(26)*	(7)*	(20/Oct/20)*	(Protocol deviation)*

Table 2. Concomitant medication

Subject#	Medication (sales name)	Indication	Start date	End date or «in progress»
13	Immodium®	Diarhea	29/Sep/20	29/Sep/20
27	Efferalgan®	Headache	3/Oct/20	3/Oct/20

Kinetics

The kinetics are mentioned in the Table 3.

Storage

Until the beginning of the study, products are kept at room temperature in a dedicated air-conditioned room, which is locked, and access controlled.

Attribution to the subjects and instructions

All the subjects received the same product reference, labelled «ATA Mg® 770 mg».

The boxes contained 60 capsules of 385 mg each (Mg^{2+}: 23,45 mg per capsule). The recommended directions use was 2 capsules daily (one in the morning, the other one, at night).

Questionnaire regarding PMS symptoms

The subjective questionnaire used in this clinical study is based on The Development of a Menstrual Distress Questionnaire[3,5].

Study stage

VISIT 1 (D-1 Month) (+1-3 days after the end of the menstrual cycle)

The subjects come to the investigation center, they were informed about the trial objectives, the procedures and the risks of the study with the information sheet. They signed two copies of the Consent Form, had to do a pregnancy test and fill in the preselection questionnaire.

Table 3. Design summary of the clinical study

Procedure	V1 Pre-inclusion	At home	V2 (By phone)	At home	V3	At home	V4
Intervals: Days	T-1 month 1 to 3 days after the end of menstrual cycle	Maximum 1 week before menstrual cycle	T0 1 to 3 days after the end of the 1st menstrual cycle	Maximum 1 week before menstrual cycle	T1 month 1 to 3 days after the end of the 2nd menstru al cycle	Maximum 1 week before menstrual cycle	T2 month 1 to 3 days after the end of the 3rd menstrual cycle
Written consent, Pregnancy test	●						
Medical History	●						
Previous treatments	●						
PMS symptoms questionnaire before menstrual cycle		●		●		●	
Medical interview with symptom questionnaire after menstrual cycle	●				●		●
Delivery of treatments, of questionnaire before menstrual cycle and the daily log	●				●		
Medical interview			●				
Return of treatments, questionnaire before the menstrual cycle and daily log					●		●
Events /Adverse Reactions Compliance / Concomittant treatment			●		●		●

The Doctor verified inclusion and non-inclusion criteria, selected subjects based on the preselection questionnaire; performed a clinical examination of the general health state and asked the subjects about their usual unpleasant sensations and medications.

The technician explained to the subjects the product utilization conditions and frequency; gave to the subjects the product to be used according to the instructions, the daily log to write down their possible unpleasant sensations or medications; the questionnaire regarding the PMS symptoms to be filled at home.

VISIT 2 – By phone (ON D0) (+1-3 days after the end of the first menstrual cycle)

The Doctor performed an interrogation of the general health state; asked the subjects about any unpleasant sensations and medications; interrogated the subjects based on the filled questionnaire about the PMS symptoms.

VISIT 3 ON (D+1 MONTH) (+1-3 days after the end of the second menstrual cycle)

The subjects returned to the investigation center one to three days after the second menstrual cycle and brought back the filled questionnaire regarding the PMS symptoms.

The Doctor performed a clinical examination of the general health state; asked the subjects about any unpleasant sensations and medications; interrogated the subjects based on the filled questionnaire about the PMS symptoms.

The technician gave to the subjects the product to be used according to the instructions and the questionnaire regarding the PMS symptoms to be filled at home.

VISIT 4 ON (D+2 MONTHS) (+1-3 days after the end of the third menstrual cycle)

The subjects returned to the investigation center one to three days after the third menstrual cycle; brought back the filled questionnaire regarding the PMS symptoms; brought the daily log and study product.

The Doctor performed a clinical examination on the general health state; asked the subjects about any unpleasant sensations and medications and interrogated the subjects based on the filled questionnaire about the PMS symptoms.

Results and discussion

For each of the 20 PMS symptoms[3-5], the summary of results and the statistical analysis is shown.

Legend:

°: paired t-test • μ: unpaired t-test • *: Wilcoxon signed rank test • §: Mann-Whitney test

1. Nervous tension

		ATA Mg® 770mg
V2-V1	N(miss)	19(2)
	mean(SD)	-0.26(0.45)
	p-value	0.0625*
V3-V1	N(miss)	19(2)
	mean(SD)	-0.58(1.17)
	p-value	0.0447°
V4-V1	N(miss)	19(2)
	mean(SD)	-0.89(1.05)
	p-value	0.0016°

First cycle:

• ATA Mg® 770mg induced a decrease in nervous tension (mean -0.26) as compared to baseline.

Second cycle:

• ATA Mg® 770mg induced a statistically significant decrease in nervous tension (mean -0.58) as compared to baseline.

Third cycle:

• ATA Mg® 770mg induced a statistically significant decrease in nervous tension (mean -0.89) as compared to baseline.

2. Mood swings

		ATA Mg® 770mg
V2-V1	N(miss)	19(2)
	mean(SD)	-0.58(0.69)
	p-value	0.0059*
V3-V1	N(miss)	19(2)
	mean(SD)	-1.16(1.17)
	p-value	0.0004°
V4-V1	N(miss)	19(2)
	mean(SD)	-1.42(1.07)
	p-value	<.0001*

First cycle:

• ATA Mg® 770mg induced a statistically significant decrease in mood swings (mean -0.58) as compared to baseline.

Second cycle:

• ATA Mg® 770mg induced a statistically significant decrease in mood swings (mean -1.16) as compared to baseline.

Third cycle:

• ATA Mg® 770mg induced a statistically significant decrease in mood swings (mean -1.42) as compared to baseline.

3. Irritability

		ATA Mg® 770mg
V2-V1	N(miss)	19(2)
	mean(SD)	-0.37(0.50)
	p-value	0.0156*
V3-V1	N(miss)	19(2)
	mean(SD)	-0.89(1.10)
	p-value	0.0023°
V4-V1	N(miss)	19(2)
	mean(SD)	-1.21(0.98)
	p-value	<.0001°

First cycle:

• ATA Mg® 770mg induced a statistically significant decrease in irritability (mean -0.37) as compared to baseline.

Second cycle:

• ATA Mg® 770mg induced a statistically significant decrease in irritability (mean -0.89) as compared to baseline.

Third cycle:

• ATA Mg® 770mg induced a statistically significant decrease in irritability (mean -1.21) as compared to baseline.

4. Anxiety

First cycle:

• ATA Mg® 770mg induced a statistically significant decrease in anxiety (mean -0.42) as compared to baseline.

Second cycle:

• ATA Mg® 770mg induced a statistically significant decrease in anxiety (mean -0.84) as compared to baseline.

Third cycle:

• ATA Mg® 770mg induced a statistically significant decrease in anxiety (mean -1.21) as compared to baseline.

		ATA Mg® 770mg
V2-V1	N(miss)	19(2)
	mean(SD)	-0.42(0.61)
	p-value	0.0156*
V3-V1	N(miss)	19(2)
	mean(SD)	-0.84(1.42)
	p-value	0.0190°
V4-V1	N(miss)	19(2)
	mean(SD)	-1.21(0.98)
	p-value	<.0001°

5. Feeling of loneliness

		ATA Mg® 770mg
V2-V1	N(miss)	19(2)
	mean(SD)	-0.32(0.89)
	p-value	0.2188*
V3-V1	N(miss)	19(2)
	mean(SD)	-0.68(0.95)
	p-value	0.0055°
V4-V1	N(miss)	19(2)
	mean(SD)	-0.89(0.74)
	p-value	0.0002*

First cycle:

• ATA Mg® 770mg induced a decrease in the feeling of loneliness (mean -0.32) as compared to baseline.

Second cycle:

• ATA Mg® 770mg induced a statistically significant decrease in the feeling of loneliness (mean -0.68) as compared to baseline.

Third cycle:

• ATA Mg® 770mg induced a statistically significant decrease in the feeling of loneliness (mean -0.89) as compared to baseline.

6. Headache

		ATA Mg® 770mg
V2-V1	N(miss)	19(2)
	mean(SD)	-0.47(0.70)
	p-value	0.0195*
V3-V1	N(miss)	19(2)
	mean(SD)	-0.95(1.13)
	p-value	0.0018°
V4-V1	N(miss)	19(2)
	mean(SD)	-1.26(1.10)
	p-value	<.0001°

First cycle:

• ATA Mg® 770mg induced a statistically significant decrease in headache (mean -0.47) as compared to baseline.

Second cycle:

• ATA Mg® 770mg induced a statistically significant decrease in headache (mean -0.95) as compared to baseline.

Third cycle:

• ATA Mg® 770mg induced a statistically significant decrease in headache (mean -1.26) as compared to baseline.

7. Craving for sweets

		ATA Mg® 770mg
V2-V1	N(miss)	19(2)
	mean(SD)	-0.21(0.42)
	p-value	0.1250*
V3-V1	N(miss)	19(2)
	mean(SD)	-0.47(0.84)
	p-value	0.0245°
V4-V1	N(miss)	19(2)
	mean(SD)	-0.74(1.05)
	p-value	0.0107*

First cycle:

• ATA Mg® 770mg induced a decrease in cravings for sweets (mean -0.21) as compared to baseline.

Second cycle:

• ATA Mg® 770mg induced a statistically significant decrease in cravings for sweets (mean -0.47) as compared to baseline.

Third cycle:

• ATA Mg® 770mg induced a statistically significant decrease in cravings for sweets (mean -0.74) as compared to baseline.

8. Increased appetite

		ATA Mg® 770mg
V2-V1	N(miss)	19(2)
	mean(SD)	-0.47(0.84)
	p-value	0.0469*
V3-V1	N(miss)	19(2)
	mean(SD)	-0.63(1.34)
	p-value	0.0551°
V4-V1	N(miss)	19(2)
	mean(SD)	-1.05(1.08)
	p-value	0.0005°

First cycle:

• ATA Mg® 770mg induced a statistically significant decrease in increased appetite (mean -0.47) as compared to baseline.

Second cycle:

• ATA Mg® 770mg induced a decrease in increased appetite (mean -0.63) as compared to baseline.

Third cycle:

• ATA Mg® 770mg induced a statistically significant decrease in increased appetite (mean -1.05) as compared to baseline.

9. Heart pounding

		ATA Mg® 770mg
V2-V1	N(miss)	19(2)
	mean(SD)	-0.26(0.56)
	p-value	0.1250*
V3-V1	N(miss)	19(2)
	mean(SD)	-0.37(0.96)
	p-value	0.1100°
V4-V1	N(miss)	19(2)
	mean(SD)	-0.68(0.82)
	p-value	0.0039*

First cycle:

• ATA Mg® 770mg induced a greater decrease in heart pounding (mean -0.26) as compared to baseline.

Second cycle:

• ATA Mg® 770mg induced a greater decrease in heart pounding (mean -0.37) as compared to baseline.

Third cycle:

• ATA Mg® 770mg induced a statistically significant decrease in heart pounding (mean -0.68) as compared to baseline.

10. Fatigue

		ATA Mg® 770mg
V2-V1	N(miss)	19(2)
	mean(SD)	-0.47(0.77)
	p-value	0.0313*
V3-V1	N(miss)	19(2)
	mean(SD)	-0.47(1.35)
	p-value	0.1431°
V4-V1	N(miss)	19(2)
	mean(SD)	-1.21(1.08)
	p-value	0.0001°

First cycle:

• ATA Mg® 770mg induced a statistically significant decrease in fatigue (mean -0.47) as compared to baseline.

Second cycle:

• ATA Mg® 770mg induced a decrease in fatigue (mean -0.47) as compared to baseline.

Third cycle:

• ATA Mg® 770mg induced a statistically significant decrease in fatigue (mean -1.21) as compared to baseline.

11. Dizziness or faintness

First cycle

• ATA Mg® 770mg induced a statistically significant decrease in dizziness and faintness (mean -0.21) as compared to baseline.

Second cycle:

• ATA Mg® 770mg induced a statistically significant decrease in dizziness and faintness (mean -0.68) as compared to baseline.

Third cycle:

• ATA Mg® 770mg induced a statistically significant decrease in dizziness and faintness (mean -0.89) as compared to baseline.

		ATA Mg® 770mg
V2-V1	N(miss)	19(2)
	mean(SD)	-0.21(0.54)
	p-value	0.2188*
V3-V1	N(miss)	19(2)
	mean(SD)	-0.68(0.67)
	p-value	0.0010*
V4-V1	N(miss)	19(2)
	mean(SD)	-0.89(0.81)
	p-value	0.0005*

12. Depression

		ATA Mg® 770mg
V2-V1	N(miss)	19(2)
	mean(SD)	-0.11(0.46)
	p-value	0.6250*
V3-V1	N(miss)	19(2)
	mean(SD)	-0.32(0.75)
	p-value	0.1484*
V4-V1	N(miss)	19(2)
	mean(SD)	-0.53(0.70)
	p-value	0.0078*

First cycle

• ATA Mg® 770mg induced a decrease in depression (mean -0.11) as compared to baseline.

Second cycle:

• ATA Mg® 770mg induced a decrease in depression (mean -0.32) as compared to baseline.

Third cycle:

• ATA Mg® 770mg induced a statistically significant decrease in depression (mean -0.53) as compared to baseline.

13. Forgetfulness

First cycle

• ATA Mg® 770mg induced a decrease in forgetfulness (mean -0.26) as compared to baseline.

Second cycle:

• ATA Mg® 770mg induced a statistically significant decrease in forgetfulness (mean -0.47) as compared to baseline.

Third cycle:

• ATA Mg® 770mg induced a statistically significant decrease in forgetfulness (mean -0.68) as compared to baseline.

		ATA Mg® 770mg
V2-V1	N(miss)	19(2)
	mean(SD)	-0.26(0.65)
	p-value	0.1875*
V3-V1	N(miss)	19(2)
	mean(SD)	-0.47(0.84)
	p-value	0.0469*
V4-V1	N(miss)	19(2)
	mean(SD)	-0.68(0.75)
	p-value	0.0020*

14. Crying

		ATA Mg® 770mg
V2-V1	N(miss)	19(2)
	mean(SD)	-0.32(0.82)
	p-value	0.1875*
V3-V1	N(miss)	19(2)
	mean(SD)	-0.53(1.12)
	p-value	0.0859*
V4-V1	N(miss)	19(2)
	mean(SD)	-0.84(1.01)
	p-value	0.0020*

First cycle:

• ATA Mg® 770mg induced a statistically significant decrease in crying (mean -0.32) as compared to baseline.

Second cycle:

• ATA Mg® 770mg induced a statistically significant decrease in crying (mean -0.53) as compared to baseline.

Third cycle:

• ATA Mg® 770mg induced a statistically significant decrease in crying (mean -0.84) as compared to baseline.

15. Confusion

		ATA Mg® 770mg
V2-V1	N(miss)	19(2)
	mean(SD)	-0.32(0.89)
	p-value	0.2188*
V3-V1	N(miss)	19(2)
	mean(SD)	-0.53(0.70)
	p-value	0.0078*
V4-V1	N(miss)	19(2)
	mean(SD)	-0.79(0.85)
	p-value	0.0010*

First cycle:

• ATA Mg® 770mg induced a greater decrease in confusion (mean -0.32) as compared to baseline.

Second cycle:

• ATA Mg® 770mg induced a greater decrease in confusion (mean -0.53) as compared to baseline.

Third cycle:

• ATA Mg® 770mg induced a statistically significant decrease in confusion (mean -0.79) as compared to baseline.

16. Insomnia

		ATA Mg® 770mg
V2-V1	N(miss)	19(2)
	mean(SD)	-0.42(0.69)
	p-value	0.0313*
V3-V1	N(miss)	19(2)
	mean(SD)	-0.63(1.12)
	p-value	0.0239°
V4-V1	N(miss)	19(2)
	mean(SD)	-0.95(0.78)
	p-value	0.0002*

First cycle:

• ATA Mg® 770mg induced a statistically significant decrease in insomnia (mean -0.42) as compared to baseline.

Second cycle:

• ATA Mg® 770mg induced a statistically significant decrease in insomnia (mean -0.63) as compared to baseline.

Third cycle:

• ATA Mg® 770mg induced a statistically significant decrease in insomnia (mean -0.95) as compared to baseline.

17. Weight gain

		ATA Mg® 770mg
V2-V1	N(miss)	19(2)
	mean(SD)	-0.21(0.42)
	p-value	0.1250*
V3-V1	N(miss)	19(2)
	mean(SD)	-0.47(0.77)
	p-value	0.0313*
V4-V1	N(miss)	19(2)
	mean(SD)	-0.53(0.96)
	p-value	0.0286°

First cycle:

• ATA Mg® 770mg induced a statistically significant decrease in weight gain (mean -0.21) as compared to baseline.

Second cycle:

• ATA Mg® 770mg induced a statistically significant decrease in weight gain (mean -0.47) as compared to baseline.

Third cycle:

• ATA Mg® 770mg induced a statistically significant decrease in weight gain (mean -0.53) as compared to baseline.

18. Swelling of extremities

		ATA Mg® 770mg
V2-V1	N(miss)	19(2)
	mean(SD)	-0.21(0.79)
	p-value	0.4063*
V3-V1	N(miss)	19(2)
	mean(SD)	-0.53(0.77)
	p-value	0.0176*
V4-V1	N(miss)	19(2)
	mean(SD)	-0.74(0.99)
	p-value	0.0045°

First cycle:

• ATA Mg® 770mg induced a decrease in swelling of extremities (mean -0.21) as compared to baseline.

Second cycle:

• ATA Mg® 770mg induced a statistically significant decrease in swelling of extremities (mean -0.53) as compared to baseline.

Third cycle:

• ATA Mg® 770mg induced a statistically significant decrease swelling of extremities (mean -0.74) as compared to baseline.

19. Breast tenderness

		ATA Mg® 770mg
V2-V1	N(miss)	19(2)
	mean(SD)	-0.74(0.81)
	p-value	0.0020*
V3-V1	N(miss)	19(2)
	mean(SD)	-1.21(1.03)
	p-value	<.0001°
V4-V1	N(miss)	19(2)
	mean(SD)	-1.53(0.77)
	p-value	<.0001*

First cycle:

• ATA Mg® 770mg induced a statistically significant decrease in breast tenderness (mean -0.74) as compared to baseline.

Second cycle:

• ATA Mg® 770mg induced a statistically significant decrease in breast tenderness (mean -1.21) as compared to baseline.

Third cycle:

• ATA Mg® 770mg induced a statistically significant decrease in breast tenderness (mean -1.53) as compared to baseline.

20. Abdominal bloating

First cycle:

• ATA Mg® 770mg induced a statistically significant decrease in abdominal bloating (mean -0.68) as compared to baseline.

Second cycle:

• ATA Mg® 770mg induced a statistically significant decrease in abdominal bloating (mean -0.95) as compared to baseline.

Third cycle:

• ATA Mg® 770mg induced a statistically significant decrease in abdominal bloating (mean -1.32) as compared to baseline.

		ATA Mg® 770mg
V2-V1	N(miss)	19(2)
	mean(SD)	-0.68(0.89)
	p-value	0.0034°
V3-V1	N(miss)	19(2)
	mean(SD)	-0.95(1.03)
	p-value	0.0008°
V4-V1	N(miss)	19(2)
	mean(SD)	-1.32(0.95)
	p-value	<.0001°

Undesirable effects/adverse effects

Adverse Effects were reported by 2 subjects. An episode of mild diarrhea (duration < 24h, medication name: Immodium®) was filled by Subject #13, before the first capsule intake and several hours after the first capsule intake.

A mild headache has also been reported (duration < 24h, medication name: Efferalgan®) approximately 2 hours after the first capsule intake. In both cases, the causality assessment was classified as unlikely.

Conclusion

The clinical study carried out on 19 women over 3 menstrual cycles clearly demonstrated the effectiveness of ATA Mg® on the 20 symptoms of Pre-Menstrual Syndrome.

In other words, ATA Mg® can offer an alternative to the medicinal drugs to help women who suffer from Pre-Menstrual Syndrome and who prefer to use a natural magnesium solution, during their menstrual cycles.

ATA Mg® 770mg induced a statistically significant decrease in nervous tension, mood swings, irritability, anxiety, feeling of loneliness, headache, cravings for sweets, increased appetite, heart pounding, fatigue, dizziness and faintness, depression, forgetfulness, crying, confusion, insomnia, weight gain, swelling of extremities, breast tenderness and abdominal bloating.

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Instructions for the authors

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Abstract

In this paper the instructions for preparing camera ready paper for the Journal are given. The recommended, but not limited text processor is Microsoft Word. Insert an abstract of 50-100 words, giving a brief account of the most relevant aspects of the paper. It is recommended to use up to 5 key words.

Key words: Camera ready paper, Journal.

Introduction

In order to effect high quality of Papers, the authors are requested to follow instructions given in this sample paper. Regular length of the papers is 5 to 12 pages. Articles must be proofread by an expert native speaker of English language. Can't be accepted articles with grammatical and spelling errors.

Instructions for the authors

Times New Roman 12 points font should be used for normal text. Manuscript have to be prepared in a two column separated by 5 mm. The margins for A4 (210×297 mm2) paper are given in Table 1.

Table 1. Page layout description

Paper size	A4
Top margin	20 mm
Bottom margin	20 mm
Left margin	20 mm
Right margin	18 mm
Column Spacing	5 mm

Regular paper may be divided in a number of sections. Section titles (including references and acknowledgement) should be typed using 12 pt fonts with **bold** option. For numbering use Times New Roman number. Sections can be split in subsection, which should be typed 12 pt *Italic* option. Figures should be one column wide. If it is impossible to place figure in one column, two column wide figures is allowed. Each figure must have a caption under the figure. Figures must be a resolution of 300 DPI, saved in TIFF format, width 10 cm min. For the figure captions 12 pt *Italic* font should be used. (1)

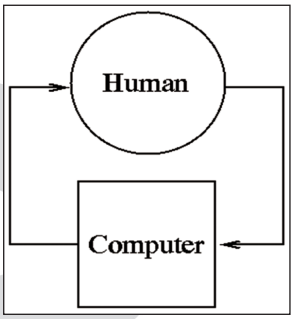

Figure 1. Text here

Conclusion

Be brief and give most important conclusion from your paper. Do not use equations and figures here.

Acknowledgements (If any)

These and the Reference headings are in bold but have no numbers.

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