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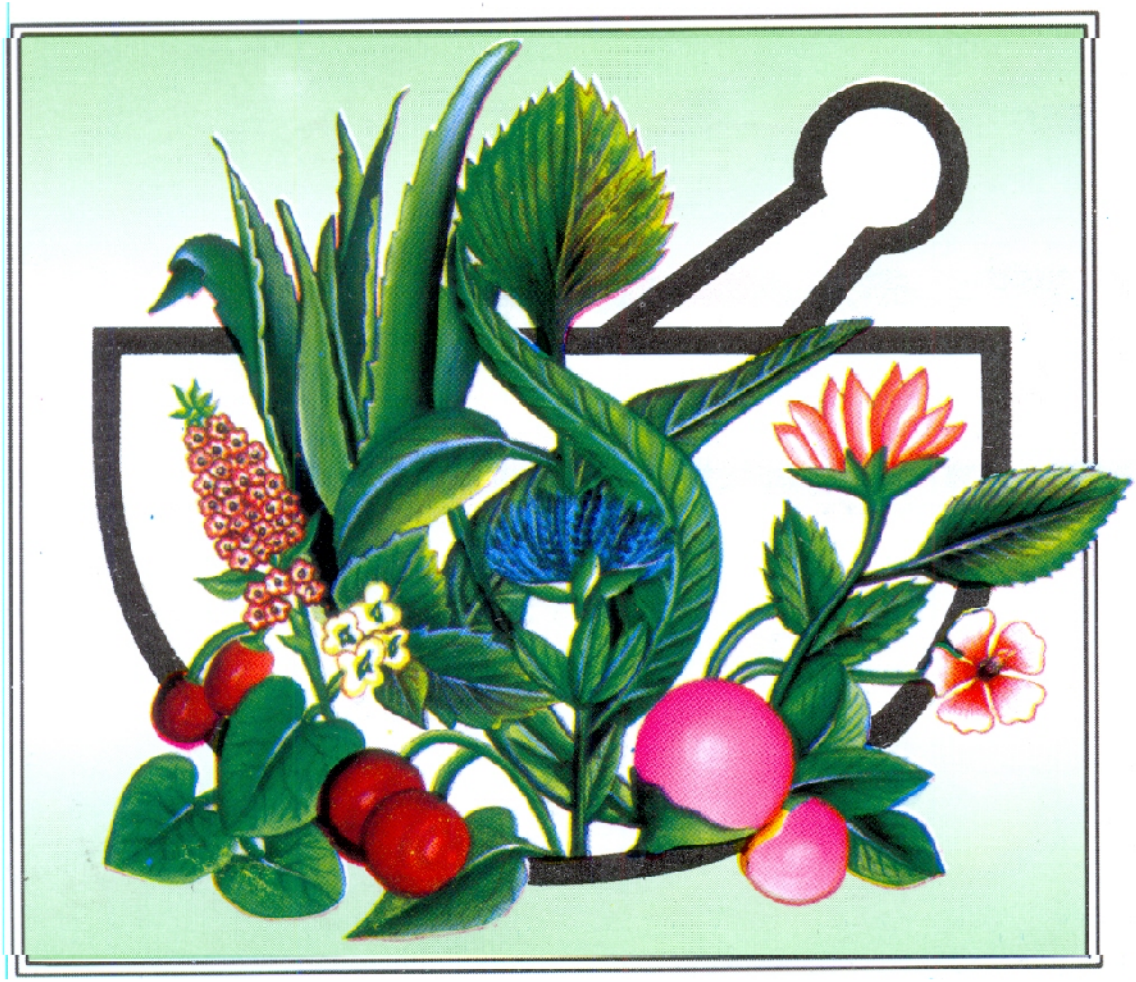

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MADINAT AL-HIKMAH
City of Education, Science and Culture

Shaheed Hakim Mohammed Said (1920-1998), a scion of the renowned South Asian Hamdard family, decided in 1948 to make the newly created Pakistan his home. He settled down in Karachi and by untiring, single minded devotion and commitment, braving all handicaps, created Hamdard Pakistan. He developed it into the leading pharmaceutical complex of Eastern Medicine in the country, run on the latest modern lines and techniques, supplying drugs of high quality and purity, backed with free clinical consultations to help ailing humanity. Hamdard Pakistan, under his leadership, also emerged as the leading philanthropic organization, and also tried to motivate people through dialogue, conferences, and journals like the *Hamdard Medicus*. In addition, he provided help to various institutions and academic bodies.

He was restless to do more, and during one of his *Hajj* pilgrimages, he envisioned the creation of a comprehensive City of Education, Science and Culture: the Madinat al-Hikmah. Work on it was initiated and funded by the Hamdard Foundation Pakistan in 1981, in the picturesque surroundings of Bund Murad Khan, 35 kilometers away from the city centre. Today it is a beehive of activity, with children and youth engaged in academic, technical and sports activities, devoted to promoting learning and culture, and through it, help achieve moral and physical welfare, peace and progress. The Bait al-Hikmah Library, Hamdard Public School, free Hamdard Village School, Centre for Horticulture and the Hamdard University are the major institutions found here. The Hamdard University's mission provides value-based education to all students in its constituent institutions. Its prestigious institutions – some based at the main campus and some in the city and some in Islamabad - include Hamdard Institute of Management Sciences (HIMS), Hamdard College of Medicine and Dentistry (HCMD), Hamdard Al-Majeed College of Eastern Medicine (HACEM), Shifa ul-Mulk Memorial Hospital, Hamdard University Hospital – Naimat Begum Mother & Child Care Unit (part of the Hamdard University Centre of Excellence), Hamdard Institute of Education and Social Sciences (HIESS), Dr Hafiz Mohammad Ilyas Institute of Pharmacology and Herbal Sciences (HMI-IPHS), Hamdard School of Law, Faculty of Pharmacy and Faculty of Engineering Sciences and Technology (FEST). The Madinat al-Hikmah continues to develop and grow.

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INFORMATION FOR CONTRIBUTORS

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Abstract: On a separate page (200-250 words) with Keywords (3-5).

The Text For Articles Should Contain: Main headings in caps and bold as:
1. INTRODUCTION, **2. MATERIALS AND METHODS** (2.1 sub-headings [in ***bold italics***] describing animals, chemicals, preparation of extract, experiments and statistical analysis), **3. RESULTS AND DISCUSSION, 4. CONCLUSION** and **ACKNOWLEDGEMENTS** (if needed) before References, **5. REFERENCES.**.
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(Articles) 1. Quinton, R. (2012). The increase in the toxicity of yohimbine induced by imipramine and other drugs in mice. *Brit. J. Pharmacol.* **21**:51-66.
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Traditional Remedy of *Morus nigra* L. Herb Formulated into Lozenges to Enhance the Localized Effect

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Abstract

Allopathic system of medication is the most popular and preferable approach of the treatment in the current scenarios, but in spite of the wide use of allopathic medicine, the trend of use of herbal medicines are also valuable contributor with the current medicinal therapies in the health care professions. In this connection, an eastern use of *Morus nigra L.* fruit was formulated into solid dosage form named as Lozenges. The lozenges consisted of *Morus nigra* L. aqueous extract, which dissolves in the mouth providing localized effect against sore throat and cough. A full-scale batch of lozenges trial was manufactured to ensure uniform process and analytical parameters.

The un-packed lozenges were evaluated for qualitative specifications like, average weight which was about 2.250-2.750 (2.5 g±10%) and uniformity of weight was 18/20-NMT ±10% 2/20. The results were found within the official limits. The appearance of the lozenges was checked organoleptically while dimensions were evaluated using calibrated vernier caliper. Packed and finished lozenges dosage form were evaluated for microbiological test like, aerobic bacteria, fungi, *P.aeruginosa*, *E. coli, Staphylococcus aureus, Salmonella* and other enteric bacteria. Thin Layer Chromatography (TLC) confirmed the presence of flavonoids as anthocyanins at 254 nm. The quantity of flavonoids as anthocyanin (cyanidin-3-glucoside) was determined as not less than (NLT) 0.80 mg/Loz. Caffeic acid was also estimated quantitatively using High Performance Thin Layer Chromatography (HPTLC) and was found NLT 0.002 mg/loz.

Keywords

Morus nigra L. Extract, Spectrophotometry, Lozenges, Flavonoids, Anthocyanins.

1. INTRODUCTION

M. nigra L, Family: Moraceae; normally known as: Black mulberry, other regional name: Tor Toot, Shahtut; Parts used are: leaves, Fruits, wood and branches; Medicinal value: laxative and purgative. Leaves have emollient and astringent properties. Fruits are used as body stimulant. It is also used as a remedy for cough and cold (Sher *et al.,* 2010). Two species are indigenous to the United States while further

Fig. 3: Image of *Morius nigra* L.

are dispersed all over the warm part of clement regions of Africa and North-America. The juice of this plant is milky and carry the fruit for which they are titled. *Morus alba*, also known as White Mulberry is famous as the principal food source for silk worms and broadly cultivate in China. One of the type also found in Eastern America as white mulberry its fruit is white to pinkish, distinct to generally available red or black berries of furthermost other species of *Morus*. The *Chinese Pharmacopoeia* (1985) register almost every part as an ingredient used for medicine preparation including leaves, root, bark, branches, and fruits. Other parts, including the sap and wood ash also widely used. *Morus nigra* L. has a valid history as per usage in Chinese medicine (Pawlowska *et al.,* 2008). *M. nigra* L fruit is a traditional Chinese medicine, effective for the remedial action of sore throat, hypertension, fever and anemia (Li *et al.,* 2009).

The Mulberry plant is also a loaded cradle of natural isoprenoid substituted phenolic compounds including flavonoids. Investigators have taken these compounds with great interest for many reasons such as structural, biological and pharmacological reasons. Series of isoprenoid can be obtained from various species of *Morus nigra* L, Substituted phenolic compounds such as Kwanon G and H which have researcher's interest for biosynthetic point of view, (Nomura and Hano, 1994). The extract prepared using methanolic method has tremendous activities for example anti-inflammatory, exudative, proliferative and anti-pyretic (Alener and Bingoul, 1988). Root bark consist of a bitter acid taste possessed cathartic and anthelmintic properties. Root is among the important sources of constituent known as, "Glucosidase" as anti-hypertensive agent. Root juice agglutinates the blood and is very useful in killing the worms in digestive system. It recommended as febrifuge, antidiarrheal, antimalaria and amaeoebiotic agents (Shivkumar *et al.,* 1995).

The stem bark is employed as purgative and vermifuge (Singh and Ghosh, 1992). Leaves juice keeps skin smooth, healthy and prevent throat infections, irritations and inflammations. The fruit is nutritive, refrigerant and laxative. The juice from fruit is like unique drink to treat convalescence from febrile diseases. The bark is purgative and vermifuge. Leaves are used in conjunctivitis. The sweet dark black juice of fruits used in sore throat: Natives prepare a mixture of fruit known as shera and is used against hepatitis (Gorsi and Miraj, 2002). *Morus* species has refrigerant and laxative properties (Venkatesh Kumar and Chauhan, 2008). According to other researcher , the traditional use of mulberry parts are effective in bad thorax and stomach worms (Ismail and Nisar, 2010).

2. MATERIALS AND METHODS

2.1 *Study Design and Duration*

The design and development of the herbal formulation consisted of the incorporation of the herbal liquid extract into the current handy modern solid dosage form, i.e. Lozenges. The study was planned, formulated and developed

with the help of literature survey which took approximately 8 weeks. The collection and verification of herbs took 6 weeks. Extraction of the herbs was carried out using deionized water. After extraction, formulation and development of lozenges (manufacturing phase) was completed within 7 weeks. Analytical evaluation using HPTLC (High Performance Thin Layer Chromatography), microbiological testing, packing and stability studies were completed within 2 weeks.

2.2. *Collection of Herbs*

The herbs of *Morus nigra* L. were purchased from local market by sourcing and procurement department of Herbion Pakistan Pvt. Limited. The herbs were also evaluated and verified by Dr. Muhammad Ishtiaq Hussain, Center for Plant Conservation Taxonomy at University of Karachi. All chemicals, reagents and reference standards used were of pure analytical grade and obtained from approved suppliers of Herbion Pakistan Pvt. Limited.

2.3. *Preparation of Extracts*

Herb is subjected to crush with interlude through grinder. The quantity of grinded herbs was accurately weighted and transferred into extractor containing deionized water (D.I.). Heating was started with continuous stirring till boiling. Once boiled, the temperature was reduced and maintained at 90-100°C for 2.5 hours. At the completion of extraction, steam was released and aqueous extract was sieved through 100 mesh size. Then the filtrate was transferred to the evaporator for further process to obtain the desired thick extract, during which the temperature was maintained at 100-110°C. Preservatives was added to the extract as per British Pharmacopeia and then mixed for 10-15 minutes. Total weight of extract was recorded for calculation purpose.

Table 1: Quality Parameters Against Standards

Parameters	Methods	Standards
Description	Organoleptically	Reddish brown to maroon red colored round shaped lozenges with characteristic taste. Slight unevenness, bubbles and whitish spots are permissible.
Identification – Flavonoids as Anthocyanins	TLC	A spot of blue violet color will be visible on the plate in UV light of wave length 254 nm.
Average lozenge weight and weight uniformity	In-house	**Average weight:** 2.250-2.750 (2.5 grams ±10%) **Weight uniformity:** 18/20 – Not more then ±10% 2/20 – Not more then ±20%
Quantitative determination	Spectrophotometric method	Flavonoids as Anthocyanins (cyanidin-3-glucoside) should be NLT 0.80 mg/Lozenge.
	HPTLC method	Caffeic acid contents should NLT 0.002 mg per lozenges

2.4. *Preparation of Syrup*

The accurately weighed quantities of deionized water, sugar and liquid glucose was transferred into double jacketed steam tank to form syrup . The temperature of the tank was maintained between 60 and 80°C. The sugar syrup was then transfered into the cooker and cooked at 148-153°C to obtain predetermined properties of hard boiled candies. Care must be taken on the parameters to keep stable vacuum. Collect the mass at the bottom up to a certain number of revolution of product pump shaft set by the operator to determine the lot size of 50 kg.

Addition of Lozenges Extract

Addition of thick extract of fruit of *Morus nigra* L. precise quantity added as per manufacturing flow diagram

Compound Preparation

Mixing of the pre-weighed micro ingredients like eucalyptus oil, flavors and menthol crystal as per formulation recipe in a separate container.

Addition of Additives and Compound

When cooked mass is condensed, additives like ascorbic acid, citric acid and flavor compound are added followed by proper mixing by mixer.

Kneading

Product mass is then transferred from bowl to kneading table through pan trolley to mix properly and achieve the hardness of product mass by using the chilled water running under the kneading table, talc powder and hydrogenated vegetable oil used as anti-adherent.

Roping and Sizing

Transfer the mass to batch roller then passed through rope sizing machine for roping suitable for die compression. Rope is set by the operator for uni-plast machine.

Compression

The sized mass is hard-pressed to provide shape of lozenges in to compression machine and maintained the In-house specification mentioned Table 2.

Hardening Process

The Lozenges are passed over conveyer of cooling tunnel for certain time to get the anticipated solidity.

Formulation Description

Lozenge avg. weight and weight uniformity:

Average weight: From 2.375 to 2.625 g (2.5 g ±5%)

Weight uniformity: 18/20 – NMT ±5% 2/20 – NMT ±10%.

Batch Formulation Recipe

Each Lozenge contains active *Morus nigra*. L Extract (Mono Herbal), major excipients and micro excipients.

Excipients

Bulking and Sweetening agent: Glucose syrup (BP) and sugar (BP), Anti adherent: talc and hydrogenated vegetable oil. Additives and flavors which comprises of menthol, eucalyptus oil, ascorbic acid , citric acid and selected flavoring compounds.

2.5. Qualitative Identification

TLC Method

Apply 0.06 ml of solution A to chromatographic plate (5 × 10 cm) in the form of spot. Dry the plate in hot air for 5 minutes and place it in the chromatographic tank previously saturated for 1 hour with a mixture of Methanol:Water (1:1). When solvent front reaches the plate edge, take it out and dry it in drying apparatus at 100°C for 5 minutes, a spot

Table 2: Test Parameters and Specification standards

Parameters	Plan for Sampling	Specification	Test/ Method
Unpackaged Product			
Physical Appearance	1 Unit sampled: from Start, Middle and end of every lot	Reddish brown to maroon red colored lozenges with characteristic odor	Organoleptic
Thickness and Diameter	10 Unit sampled: from Start, Middle and end of every Batch	Width – 7 mm ±1 mm Diameter – 17 mm ±1 mm	Vernier caliper
Avg. Weight	1 Unit sampled: from Start, Middle and end of every lot	2.375 gm-2.625 gm	British Ph.
Weight Uniformity	Twenty (20) units of lozenges sampled from Start, Middle and end of every lot	2.5 g ±5%	British Ph.
Assay	Quantitative determination	Flavonoids as Anthocyanins (cyanidin-3-glucoside) should be NLT 0.80 mg/Lozenge.	Spectro-photometer
		Caffeic acid contents should NLT 0.002 mg per lozenges	HPTLC method
Finished Pack Product			
Physical Blister Appearance	10 units of Blisters (Start, middle and end respectively)	Comply standard	Optical
Leakage Analysis	10 units of Blisters (Start, middle and end respectively)	Zero leakage	Vacuum desiccators
Microbiological Inspection	02 units of Blisters (Start, middle and end respectively)	Category 3B	Eur. Ph.

Table 3: Details of the Herbs, Common Name, Parts Used and Extracts Dose

Name of Herb (Aqueous extract)	Common name	Part Used	Herb: extract ratio	Extract Quantity
Morus nigra. L.	Toot, Firssad, Black Mulberry	Fruit	6.4: 1	12.0 mg

Table 4: Herbs Quantities and Pharmacological Actions

Herb source	Common name	Quantity	Pharmacological Action Reported
Morus nigra. L.	Toot, Firssad, Black Mulberry	76.2 mg	Treatment of sore throat, fever, hypertension, anemia, antitussive and expectorant

*amount of herb used in the preparation of 1 lozenge.

of blue violet color will be appear on the plate at wave length 254 nm. (flavonoids as anthocyanins).

2.6. Quantitative Determination

Total Flavonoids as Anthocyanins (cyanidin-3-glucoside)

Put 4 lozenges (exact weight) into a 200 ml flat – bottomed flask, add 50 ml of distilled water and dissolve the lozenges. Transfer the whole solution in a 250 ml dividing funnel and mix. Add 50 ml of ethyl acetate and shake well for 30 minutes then separate the layer, after full division of layers, collect the upper ethyl acetate layer in a 250 ml conical flask.

Add 50 ml of ethyl acetate in aqueous layer and repeat the same procedure for two more times (3 times in total). Collect total ethyl acetate layers in same conical flask and add about 10 g of sodium sulphate, shake and filter through cotton wool in another dried conical flask. Filtered extraction steamed to dryness on water bath. Dissolve the dry residue in 10 ml of 96 % ethanol (solution A).

Transfer 5 ml of solution A in 10 ml volumetric flask and make up the volume with 96% ethanol and measure the optical density of resulting solution in a cuvette of 1cm at 520 nm using 96% ethanol as a blank.

Calculation

Anthocyanin in single lozenge contents in milligrams (mg) (X) as pigment content as cyanidin-3-glucoside is estimated as per the below mentioned formula:

$$X \frac{D \times MW \times 10 \times 10 \times 1000 \times M_0}{\varepsilon L \times M \times 5} = \frac{20000 \times D \times MW \times M_0}{\varepsilon L \times M}$$

Where:

D = Optical density

ε = Cyanidin-3-glucoside molar absorbance (26,900)

MW =Anthocyanin molecular weight (449.2)

M = Sample weight (g)

M_0 =Average weight of lozenge (g)

L = Cell path length (1 cm)

Limit: Flavonoids as Anthocyanins (cyanidin-3-glucoside) should be NLT 0.80 mg/ Lozenge.

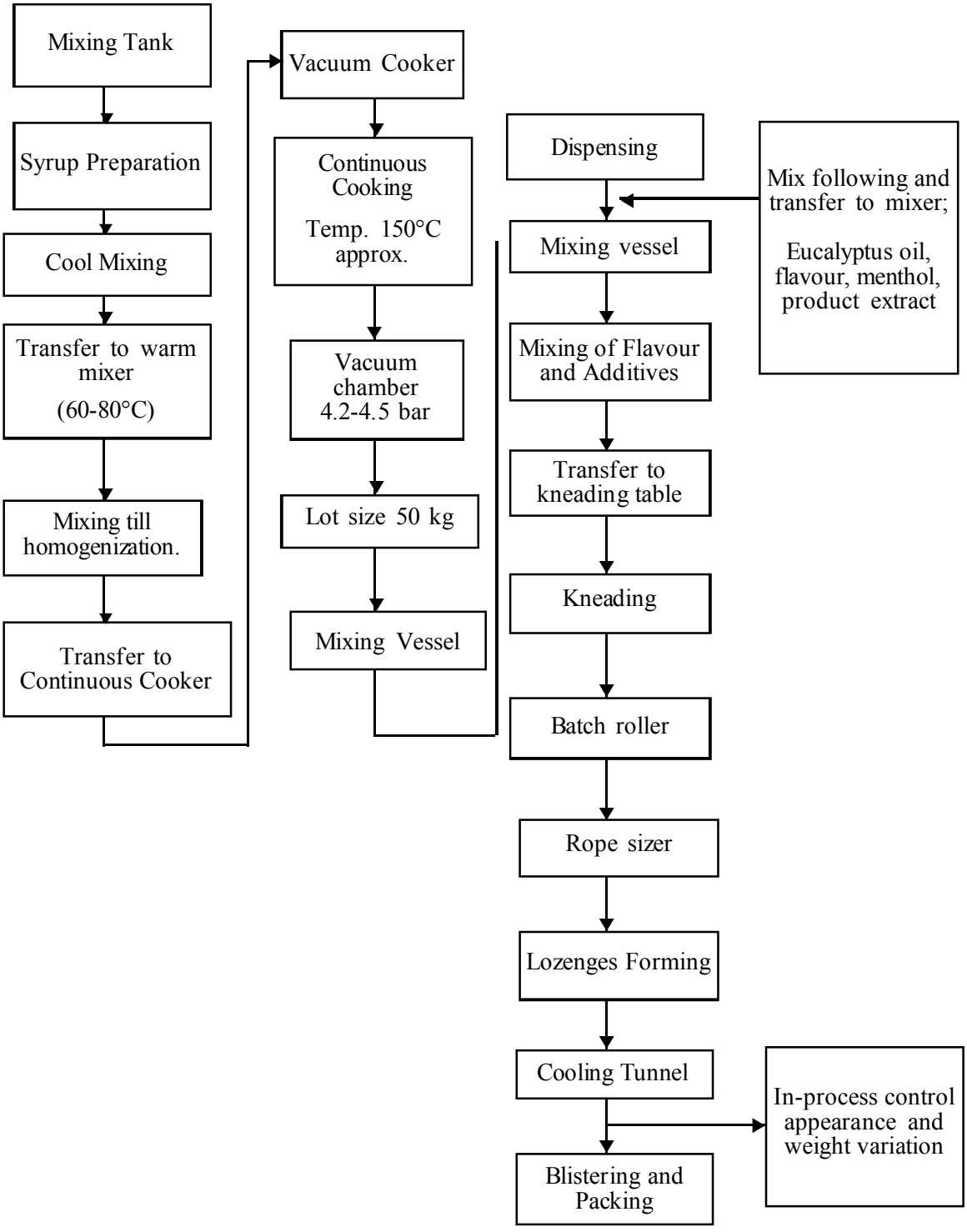

Fig. 1: Manufacturing flow diagram of Lozenges

Caffeic Acid determination by High Performance Thin Layer Chromatography

CAMAG Linomat 5, CAMAG Scanner III or Equivalent equipment can be used for the quantitative determination in which HPTLC silica gel $G_{60}F_{254}$ used with Toluene: Ethyl acetate: Formic acid solvent system observed on 326 nm UV/Wave length. Transferred approximately 1.0 mg (note exact weight) of caffeic acid standard in a 10 ml volumetric flask and dissolve in methanol as an standard and analyzed through a sample prepared by dissolving 13.0 gm crushed mono herbal lozenges (exact weight) and transferred in a 100 ml round bottom flask, added 20 ml water and dissolve the lozenges in water by sonicator. Add 20 ml methanol and 6 ml 25% hydrochloric acid; reflux the mixture for 1 hour at 100°C. After TLC preparation and development, scan the plate in the densitometer by linear scanning at 326 nm by use of a TLC Scanner III CAMAG with a mercury source and measurement mode is fluorescence. Integrate the area of the spots corresponding to Caffeic acid standard. It was concluded that Caffeic acid contents should NLT 0.002 mg per lozenges.

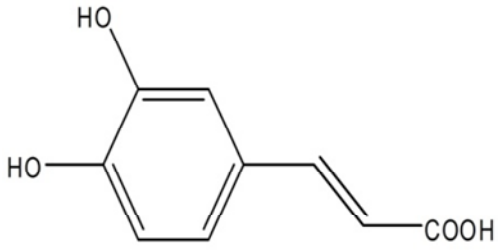

Fig. 2a: Structure of Caffeic acid

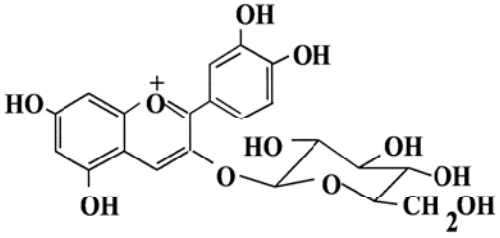

Fig. 2b: Structure of Cyanidin 3-O-β-glucoside

Microbiological Purity

Afterward to permissible content for 1 gram of preparation, the count of following should comply and must be not more then the specified limits:

aerobic bacteria – 104 cfu/g.

Fungi – 102 cfu/g,

Ps. aeruginosa, e. coli, staphylococcus aureus – absent.

Salmonella 10 grams - absent and

enteric bacteria – 102 cfu/g

Stability

Stability studies have been conducted for 6 month accelerated and ongoing real time for the evaluation of physical and chemical parameters and found compliant to standards.

3. RESULTS AND DISCUSSION

Quantitative parameters like appearance (organoleptically), weight variation, thickness and diameter including assay were incompliance with official monograph. Qualitative Assay for the determination of flavonoids as anthocyanin (cyanidin-3-glucoside) performed via TLC for quantitatively via optical density that the flavonoids as anthocyanins (cyanidin-3-glucoside) was confirmed (should be NLT 0.80 mg/Lozenge the main constituent of mono herbal lozenges with *Morus nigra* L. fruit extract as an active constituent). Another quantitative evaluation caffeic acid determination was established by High Performance Thin Layer Chromatography and content was confirmed (should be NLT 0.002 mg per lozenges.)

Multiple batches prepared to minimize the variation and for the establishment of standardization. By this practice, it was concluded that the single lozenges of *Morus nigra* L. fruit contains 12.0 mg of extract. The contemporary adjustment carried out expose adherence to complete physical chemical and analytical procedures, therefore conclusion derived is that Herbal Lozenges are customary appealing product at the base line consideration.

Morus nigra L. a single herbal extract based lozenges formulation contains the aqueous extract from fruit of plant (*Morus nigra* L.). Before the initiation, it was seasoned and established that the fruit of *Morus nigra* L. herb delivered and stored as per standards for the consistency of active biological quantifiable constituents in the mono herbal formulation with quantitative examination covers the credentials of chemical gears, however the quantitative assessment measure the credentials in addition to the intensities of biomarkers in the extract of the standard preparation. After a literature search, design and development of the lozenges were established. It was initiated by the preparation of syrup and ended at the process of compression.

Pawlowska *et al.,* identify the presence of four anthocyanins recognized as cyanidin 3-O-glucoside in *Morus nigra* L. fruits (Pawlowska *et al.,* 2008), Traditional Chinese medicine used Mulberry fruit for the treatment of sore throat, fever, hypertension, and anemia (Li *et al.,* 2009) as well as *Morus nigra* L. mulberry fruits has highest total flavonoid contents were observed in black mulberry (Ercisli and Orhan, 2007). Further more *Morus nigra* L. fruit have chemical constituents like Olcancolic acid, apigenin, cyclocommunol, morusin, cyclomorusin, kuwanon C, daucosterol, ursolic acid, 63-sitosterol (Wang *et al.,* 2007). Haq *et al.,* also recommended lozenges for the treatments of cough, as an expectorant and sore throat (Haq *et al.,* 2011).

4. CONCLUSION

The following outcome is achieved as per research objectives

Morus nigra L. herbal lozenges were developed on high-quality appearance and excellence in taste to assist a pleasant effect during the course of time to reduce the mental stress resulting from the conditions suffered by the patients. Extraordinary strength of design, expansion in natural treatment and assessment with standardize and modern analytical methods are incorporated to have confidence on the product that by transforming it from traditional preparation to modern dosage form, it carries the actives which support the efficacy of Herbal entity. HPTLC shows the actual quantity of flavonoids and anthocynanins while spectrophotometery shows the flavonoids and anthocynanins in each lozenge indicated for sore throat, cough and expectorant. By means of all pre-requisites Herbal lozenges proves the development strategy and outcome are the due carving of natural remedy.

The overall objective of this research and development activities is that how to establish *Morus nigra* L. extract in to modern dosage form as lozenges with qualitative and quantitative assessment as per recent trends of phytopharmaceuticals manufacturing.

Acknowledgements

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Conflict of Interest

The authors declare that there is no conflict of interest.

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Analgesic, Antioxidant and Cytotoxic Activity of *Calotropis gigantea* L. Latex

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Abstract

The dried latex of *Calotropis gigantea* L. was tested for its analgesic, antioxidant and cytotoxic activity in mice. The latex significantly and dose dependently inhibited acetic acid induced writhing by 79.13% and 39.4% at 500 and 250 mg/kg body weight, respectively, comparable to diclofenac sodium. The antioxidant activity was determined qualitatively based on the scavenging activity of 1,1-diphenyl-2-picryl hydrazyl (DPPH) free radical showing the presence of antioxidant principle. In brine shrimp lethality assay the latex was toxic with LC_{50} and LC_{90} values of 1.7 µg/ml and 10 µg/ml, respectively. These results suggest that latex possess analgesic and antioxidant activities supporting its traditional uses. However, *C. gigantea* latex should be used with caution as it also showed toxicity, but more experiments are required in its support

Keywords:
Calotropis gigantea L., Analgesic activity, Antioxidant activity; Brine shrimp lethality.

1. INTRODUCTION

The plant *Calotropis gigantea* L. (Family: Asclepiadaceae), abounding in milky juice is found in Bangladesh, parts of India, Sri Lanka, Singapore, Malay Islands and South China. The root bark of this plant is used as medicine in treatment of leprosy, piles, wounds, tumors, parasitic infections and dysentery (Kirtikar and Basu, 1994). The latex has emetic, diaphoretic, alterative and purgative properties. In India subcontinent, the milky juice is used illegally for inducing abortion. It is also used as a cattle poison (Biswas, 2008). Alcoholic root extract of *C. gigantea* showed analgesic, anticonvulsant, anxiolytic and sedative effect in albino rats (Argal and Pathak, 2006). Although a variety of bioactive compounds such as calotroposides A and B, two oxypregane-oligoglycosides (Kitagawa *et al.,* 1992) have

Fig. 1: *Calotropis gigantea* L. with flowers

been reported, but there is no scientific report regarding the pharmacological evaluation of the latex of this plant. The aim of the present study was to investigate the analgesic, antioxidant and toxicity of the crude latex of *C. gigantea* L.

2 Materials and Methods

2.1. *Plant Material*

The latex of *C. gigantea* L. was collected from Narail district of Bangladesh during the last week of December 2007 during the daytime. It was air dried for about one week and stored in an airtight container and kept in a dry and cool place until analysis. The plant was taxonomically identified by the expert of Bangladesh National Herbarium, Mirpur, Dhaka) and deposited as voucher specimen (accession No. DACB-32085).

2.2. *Animals*

Mice (Swiss Webster) of both sex weighing 20-25 g acquired from animal resources branch of the International Center for Diarrheal Disease and Research, Bangladesh (ICDDR) were used for experiments. Prior to experiments, animals were kept at animal house (Pharmacy Discipline, Khulna University, Khulna) for adaptation under standard laboratory conditions (relative humidity 55-65%, room temperature 25.0±2°C with 12 h. light: dark cycle) and had free access to standard diets (ICDDR formulation) tap water. *Artemia salina* (Brine shrimp) cysts were obtained from Oriental Fish Processing Industry, Khulna. One tea spoon of cysts was added to saline water and after 48 h they hatched into nauplii. Saline used was prepared by dissolving pure NaCl (20 g) and normal edible NaCl (18 g) in water (1 L).

2.3 *Acetic Acid Induced Writhing Assay*

The method of Whittle (1964) and Ahmed *et al.* (2004) was adopted for evaluation of analgesic activity with minor modifications. The experimental animals were randomly divided into four groups, each consisting of five animals. Group I: Control received 1% (v/v) Tween-80 in water at the dose of 10 ml/kg of body weight; Group II: Positive control was given diclofenac sodium 25 mg/kg (Square Pharmaceuticals Ltd., Bangladesh). Group III and IV: Test groups were treated with the latex of *C. gigantea* at 250 and 500 mg/kg, respectively. All the animals were administered orally, 30 minutes prior to the intraperitoneal injection of acetic acid (0.7%); then after interval of 15 minutes prior of writhes (squirms) were counted for 5 minutes. The number of writhes in the control was taken as 100% and percent inhibition was calculated as show below:

Inhibition of writhes (%) = 100 – (treated mean group/control mean group) × 100

2.4 *Antioxidant Activity*

Antioxidant activity of the *C. gigantea* latex was determined qualitatively on the basis of its scavenging activity of the stable DPPH free radical (Sadhu *et al.,* 2003). Suitably diluted stock solutions were spotted on pre-coated silica gel TLC plates which were developed in solvent systems of different polarities (polar, medium polar and non-polar) to resolve polar and non-polar components of the extract. The plates were dried at room temperature and were sprayed with DPPH (0.02%) in ethanol. Bleaching of DPPH by the resolved bands was observed for 10 minutes and the colour changes (yellow on purple background) were noted.

2.5 *Brine Shrimp Lethality Assay*

The method of Meyer *et al.,* (1982) was adopted to study the general toxicity of the latex

of *C. gigantea* (CG). The brine shrimp eggs were hatched in a conical flask containing brine shrimp medium (300 ml). The flask were well aerated with the aid of an air pump, and kept in a water bath at 29-30°C with provision of continuous supply with bright light. The nauplii hatched within 48 h. The dried latex was dissolved in DMSO (5%) to obtain concentration of 1, 2, 5, 10, 20 and 40 µg/ml. Each preparation was transferred into clean test tubes (10 ml) in duplicates. Appropriate control and test samples were prepared. After labelling test tubes properly, shrimps (n=10) were added to each of test tubes with the help of a Pasteur pipette. The test tubes without sample (control) and containing the sample were incubated at 29°C for 24 h. in a water bath, after which each tube was examined and the surviving brine shrimps were counted and recorded. The percentage of mortality was calculated at each concentration.

2.6 *Statistical Analysis*

The data was evaluated to determine differences between control and experimental groups using Student's t-test.

3. Results and Discussion

Analgesic activity of latex of *C. gigantea* was tested by acetic acid induced writhing model in mice. This model represents pain sensation by triggering localized inflammatory response. It is used to induce writhing causing discomfort by liberation of endogenous substances, which in turn excite the pain nerve endings (Taesotikul *et al.,* 2003). Increased levels of prostaglandins (PGE_2 and PGF_{20}) in the peritoneal fluid have been reported to be responsible for pain sensation caused by intraperitoneal administration of acetic acid (Derardt *et al.,* 1980). The frequency of induced writhes in mice were significantly and dose dependently suppressed after treatment with latex of

Table 1: Effect of *C. gigantera* Latex on Acetic Acid Induced Writhing in Mice

Animal Group	Dose (mg/kg p.o.)	Number of Writhes (% writhes)	Inhibition of writhes (%)
Control	Tween-80 1% water	29.9±1.94 (100)	
Positive control (Diclofenac sodium)	25	13.3±16 (44.78)	55.2**
Calotropis gigantea Latex	250	18±1.17 (60.60)	39.4*
	500	6.2±0.65 (20.87)	79.13**

Values are expressed as Mean ± SEM; n = number of mice; Percent writhes in control = (100)
*p<0.01; **p<0.001 vs. control; p.o. = per oral.

Table 2: Effect of on Brine Shrimp Lethality Bioassay

C. gigantean latex	Number of live shrimps	Mortality (%)	LC_{50} (µg/ml)
1.0	6	40	1.7
2.5	4	60	
5.0	3	70	
10.0	1	90	
20.0	0	100	
40.0	0	100	

C. gigantea. At the dose 250 mg/kg the latex of CG showed 39.4% inhibition ($P<0.01$), whereas at 500 mg/kg produced 79.13% of writhes inhibition ($P<0.001$). The standard drug diclofenac-Na demonstrated 55.2% writhing inhibition and all the results were statistically significant (Table 1). On the basis of these results, it appears that the dried latex of *C. gigantea* possesses analgesic activity probably via peripheral mechanism, however, experiments are required to identity the receptor(s) for the said action. Furthermore, role of CNS of also needs to be clarified before reaching any definite conclusion.

The free radical scavenging property reflecting antioxidant properties is possibly one of the mechanisms by which the latex of this plant is effective in traditional medicine. When the plate was viewed after applying DPPH on the TLC plate, yellow colour was formed indicating the presence of antioxidant principle(s) in the latex. This colour change may be due to the presence of phenolic compounds (tannins and flavonoids) residing in it (Larson, 1988).

Brine shrimp lethality bioassay is popularly used as preliminary toxicity test which has also been proposed that it does not indicate any general toxicity but is also provides information of the compounds for wide range of pharmacological activities such as antimicrobial, pesticidal, antitumor, etc. (Meyer, 1982; McLaughlin *et al.,* 1988). The latex of *C. gigantea* showed significant activity against the brine shrimp nauplii with LC_{50} and LC_{90} of 1.7 µg/ml and 10 µg/ml, respectively (Table 2). Thereby, suggesting its toxicity however, further investigations on rodents are necessary to confirm it. The identification of compounds responsible for analgesic activity and its mechanism of action needs to be explored to provide scientific justification traditional use of *C. gigantea* needs to identified that would allow to understand its mechanism of action.

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Genotoxic Evaluation of Linkus Polyherbal Cough Preparation in Bone Marrow of Wistar Rats

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Abstract

A polyherbal Linkus cough syrup is popularly used against various respiratory ailments, however there is no information regarding its genotoxicity. Thus, Linkus extract (1 and 5 g /kg) was administered orally to Wistar rats (n=9-12) for 3, 7 and 14 consecutive days with 24h intervals. Negative and positive control animals received water or cyclophosphamide (20 mg/kg), respectively. At the end of experiment, rat femora were removed and marrow was collected in Hanks balanced salt solution, smeared onto glass slides and fixed in methanol (100%). After drying, stained with Giemsa (5%) followed by the addition of DPX [A mixture of distyrene (a polystyrene), a plasticiser (tricresyl phosphate) and xylene] and observed under microscope at 100× magnification. In all the slides, normochromatic erythrocytes (NCE) and polychromatic erythrocytes (PCE) with and without micronuclei were identified; their frequency was noted and photographed. In cyclophosphamide treated animals, number of both PCE and NCE with micronuclei increased significantly as compared to control indicating its genotoxic action. On the contrary, in the Linkus treated (1 and 5 g/kg) animals the frequency of micronuclei was similar to that of control group. Since, there was no difference in the duration of treatment with Linkus at 1 and 5 g/kg, the data was pooled. Additionally, mice (n=10/sex) treated orally with either 1 or 5 g/kg of Linkus showed neither behavioral changes nor mortality for a period of 1 week.

It is concluded that Linkus (5 g/kg) in acute toxicity test is non-toxic and in rat bone marrow test it did not induce micronuclei formation and hence is non-genotoxic, thereby supporting its safe usage in humans.

Keywords

Polyherbal Linkus cough syrup, Micronucleus, Genotoxicity test.

1. INTRODUCTION

According to the World Health Organization ~80% of the population from less developed countries rely on traditional health care system predominantly herbal medicine such as Ayuruvedic, Traditional Chinese Medicine and Unani medicine which are often considered safe. Realizing the growing popularity of herbal products, the Organization of Economic Cooperation Development (OECD, 1983, 1997) has introduced the guidelines before they are marketed. Such pre-requsites for herbal products include battery of *in vitro* and *in vivo* toxicological tests including rodent miconucleus genotoxicity test which is useful and reliable to

determine their safety for human consumption and is gaining popularity.

Linkus, a polyherbal cough syrup is commonly used in Pakistan since 33 years and elsewhere with promising alleviating properties against flu, cough, asthma, cold,bronchitis and other upper and lower respiratory tract infections and is also an expectorant and anti-spasmodic (Nawaz *et al.,* 2014). It is a mixture of ten medicinal plants *viz. Adhatoda vasica, Glycyrrhiza glabra, Piper longum, Viola odorata, Hyssopus officinalis, Alpinia galangal, Cordia latifolia, Althaea officinalis, Zizyphus vulgaris* and *Onosma bracteatum* (Table 1) with *A. vasica* being most predominant plant (~40%) of the total composition. During respiratory ailments, it is equally popular and effective among adults, children and infants but differs as far as recommended dosages and duration of Linkus

Table 1: Linkus Syrup Composition

S.No.	Name of medicinal plants	mg/10 ml	(%)
1.	*Adhatoda vasica*	600	40
2.	*Glycyrrhiza glabra*	400	25
3.	*Piper longum*	100	6
4.	*Cordia latifolia*	100	6
5.	*Althaea officinalis*	100	6
6.	*Zizyphus vulgaris*	100	6
7.	*Onosma bracteatum*	100	6
8.	*Hyssopus officinalis*	50	3
9.	*Alpinia galanga*	50	3
10.	*Viola odorata*	25	1.5

The percentage of individual plant present in total of 1625 mg /10 mL is represented as percentage (%).

Table 2: Dosage and Consumption of Linkus Cough Syrup During Respiratory Ailments in Adults, Children and Infants

S.No.	Age group	Dosage	Frequency per day	Consumption volume after dosage regimen of Linkus (ml)		
				3 days	7 days	14 days
1.	Adults	2 teaspoons (10 ml)	3-4	120	280	560
2.	Children	1 teaspoon (5 ml)	3-4	60	140	280
3.	Infants	½ teaspoon (2.5 ml)	3-4	30	70	140

syrup is concerned (Table 2). Previously, preclinical studies demonstrated that it is non-toxic to rats in both acute (5 g/kg) as well as in repeated dose toxicity for 2 weeks (500 mg/kg) with no adverse effects on either hematological parameters or liver function enzymes. The citric acid-induced cough in rats was also attenuated by Linkus aqueous extract (300 mg/kg) favouring its anti-tussive properties (Nawaz *et al.,* 2014).

Indeed, toxicological evaluation of medicinal plants are important in order to ascertain their safety but now-a-days both regularity and non-regulatory bodies such as OECD, (1983), EEC (1992) and US EPA (1990) emphasize on evaluation of herbal products. In this regard the guidelines to conduct such assays have been published. Genotoxic substances (genotoxins) are potentially known to be mutagenic or carcinogenic. In humans, DNA damage or genotoxicity may be caused by exposure to exogenous agents or substances, including herbal medication. On the other hand, endogenous sources of toxins could also results from free radical action generated during diseased state. Nevertheless, the exposure of cells to genotoxic substances damages chromosomes or components of mitotic spindle leading to the formation of micronuclei. These may be formed from chromosomal material fragmented during mitosis which fail to be incorporated in daughter nuclei at completion of telophase stage of mitotic cell division. Micronuclei are coated with nuclear envelope and their DNA is often transcriptionally active that undergoes replication (Nusse, 1981; Kramer *et al.,* 1990).

The rodent bone marrow micronucleus test is widely used *in vivo* assay for the assessment of genotoxic potential of herbal products and both regularity and non-regulatory bodies such as OECD, (1983), EEC (1992) and US EPA (1990) have published the guidelines to conduct it. This assay provides a simple and reliable test in which immature bone marrow erythrocytes are used to determine mutagenic potential of chemical substances (Bakare *et al.,* 2009). Genotoxicity assessment of a pharmaceutical effluent using four bioassays (Adeoye *et al.,* 2015). Micronuclei appear during mitosis as a small, round nuclear bodies 1/3rd the size of nucleus arising from acentric fragments, or whole chromosomes, that fail to be incorporated into the daughter cells. Their appearance in the cytoplasm of interphase daughter cells has been used to quantify the clastogenic or aneugenic chromosomal DNA damages (Fenech *et al.,* 2011). Keeping in mind the importance of genotoxic evaluation of herbal products the present study was undertaken and Linkus was assessed using rodent micronucleus assay.

2. MATERIALS AND METHODS

2.1. *Procurement of Plants and Preparation of Linkus Extract*

All the aforementioned plants in Linkus were purchased from local market, identified by taxonomist, cleaned and appropriate part was separated and crushed individually and subjected to grinding followed by extraction. The Linkus syrup contain plant extracats mentioned earlier along with expedients such as sucrose anhydrous, citric acid, glycerin, methyl paraben, propyl paraben, propylene glycol, peppermint oil, clove oil and deionized water (Sheikh *et al.,* 2014). Linkus extract (1 g) in methanol (25 mL) was sonicated for about 30 minutes in the ultra sonic bath, followed by filtration through a filter with pore size of 0.45 µm. The filtrate was stored at 25±2ºC for toxicity and genotoxicity studies or immediately subjected to high pressure liquid chromatography (HPLC).

2.2. *Chemicals and Equipments*

Methanol, phosphoric acid, Giemsa stain and DPX [A mixture of distyrene (a polystyrene), a plasticiser (tricresyl phosphate) and xylene] (Merck) while pentothal sodium and cyclophosphamide were obtained from local market. Microscope (MoticBA210), HPLC (Agilent 1260 HPLC System).

2.3. *Animals*

NMRI mice (25-35g, 6 months) and Wistar (150 g to 250 g, age 5-7 months) of both sex housed at animal facility (Herbion [Pvt.] Ltd., Karachi, Pakistan) under controlled environmental conditions (25±1°C, relative humidity ~60%) with 12h light/dark cycle were used. All the animals received normal rodent food and tap water *ad libidum* and regular veterinary doctor visits confirmed that the animals were healthy and can be used for experiments.

2.4. *Toxicity Study*

The following studies were conducted to evaluate the toxicity of Linkus product in rodents:

2.4.1. *Acute toxicity*

Mice of either sex (n=10/sex) were treated orally with either 1 or 5 g/kg of Linkus. Behavioral changes and mortality were observed for a period of 1 week.

2.4.2. *Micronucleus assay*

Wistar rats (n=9-12) were weighed prior to the oral administration of either tap water or Linkus extract (1 or 5 g/kg) for 3, 7 and 14 consecutive days. They were examined after each dose for any behavioral changes for toxic effects and /or mortality. Four rats received cyclophosphamide (20 mg/kg) orally serving as positive control and sacrificed after 42h of treatment following the international guidelines. (Guidance for industry (S2 (R1) genotoxicity testing and data interpretation for pharmaceuticals intended for human use).

Harvesting of Bone Marrow

At the end of experiment rats were weighed after cervical dislocation both the femora were removed immediately followed by making a small opening at the iliac end. A syringe needle (3G) containing few drops of Hanks balanced salt solution (pH=7.4) was inserted at its epiphysial end pushing into the marrow canal. The marrow was placed directly onto a pre-cleaned glass slide and small samples were transferred to glass slides, smeared, air-dried, labeled and fixed in methanol (100%) for 5 minutes and left at room temperature. After drying, stained with Giemsa (5%) for 10 minutes followed by addition of DPX and covered with glass cover slips. These slides were observed under light microscope and photographed at 100×.

Scoring of Micronucleus

Micronuclei were identified according to the criteria established by Schmid (1975) as darkly stained (purplish), round or almond shaped with sharp boundries occurring in polychromatic and euchromatic erythrocytes.

The frequency of micronucleus was determined by counting the number of micronucleated polychromatic (PCE, ~2400, n=10) and normochromatic erythrocytes (NCE, ~289-394, n=10) and expressed as percent micronucleated PCE and NCE (Fenech, 2000).To evaluate the cytotoxic effect of Linkus the number of PCE and NCEs was counted and the PCE:NCE ratio was determined (Ouanes *et al.,* 2003).

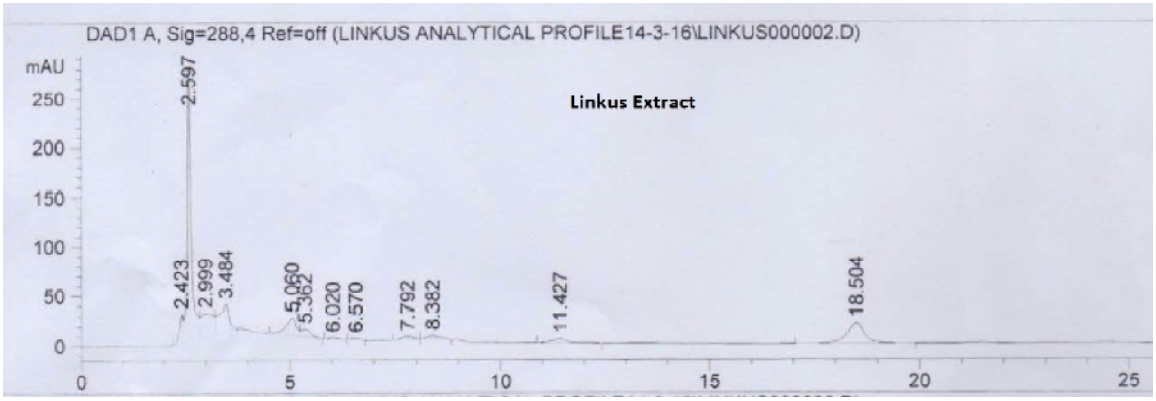
2.4.3. *Staistical Analysis*

The micronucleus data in the treated group

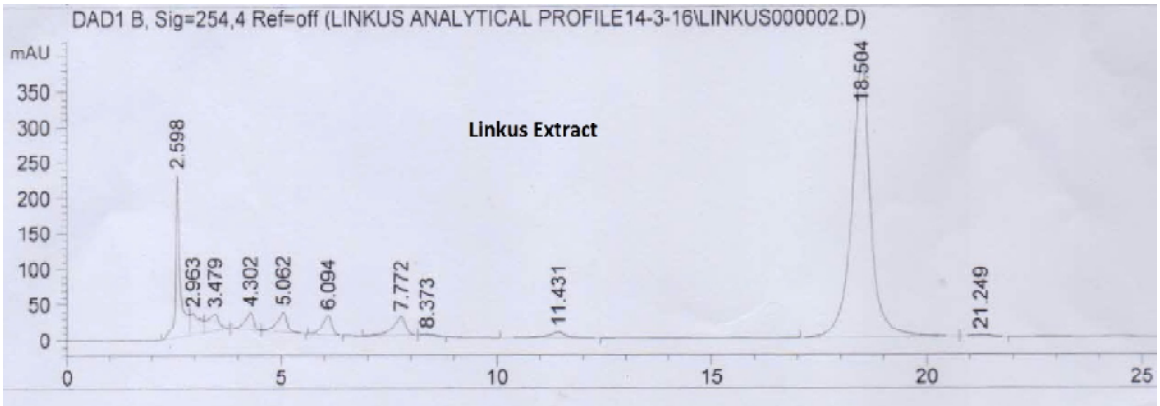
(Linkus and CP) was compared with control using ANOVA using SPSS/10 software; p value of <0.05 represented statistical significance. All the results are expressed as mean ± SEM for 4-12 animals in each group.

3. RESULTS AND DISCUSSION

As presented in Table 1, Linkus preparation predominantly contains *A. vasica* Nees (40%), followed by *G. glabra* L. (25%), *A. officinalis* L., *C. latifolia, O. bracteatum* Wall., *P. longum* L. and *Z. vulgaris* Lam. (6.2%), *A. galanga* L., *H. officinalis* L. (3%) with only 1.5% of *V. odorata* L. Each plant has multiple pharmacological properties that are accountable for their presence in Linkus preparation and justify their action against respiratory diseases e.g. *A. vasica* possesses bronchodilatory, anti-tussive, expectorant, anti-inflammatory, anti-microbial, anti-allergic, anti-asthmatic and anti-oxidant properties which are mainly associated with the alkaloid like vasicine (Claeson *et al.,* 2000).The finger print of the extract used in the study is shown as Figs. 1a and 1b clearly illustrating the presence vasicine as one of the active moiety of *A. vasica* in the Linkus extract and in Linkus syrup with a distinct peak at 254 nm with a retention time of 18.504 and 18.533, respectively confirming its presence and also facilitated its standardization.

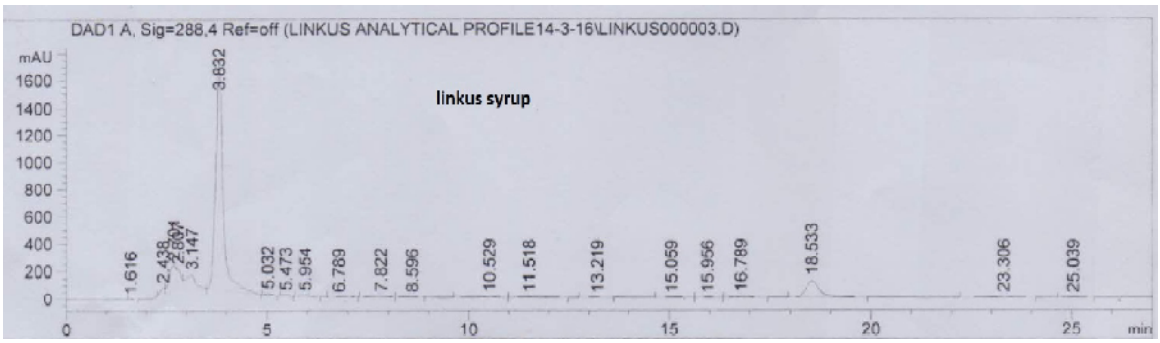


i) Wave length 288 nm



ii) Wave length 254 nm

Fig. 1a: Chromatographic profile of Linkus extract at two different wave lengths



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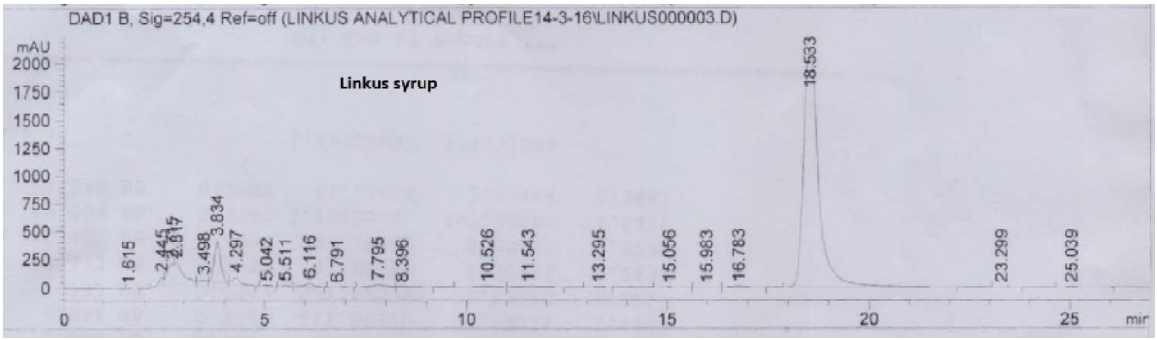

ii) Wave length 254 nm

Fig. 1b: Chromatographic profile of Linkus syrup at two different wavelengths

Fig. 1: HPLC was performed using Agilent 1260 HPLC system (Agilent Technologies, Germany). The chromatogram peaks were quantified by means of PC based (Agilent Chem Station). Chromatography separation were achieved on Phenomenex Luna 5µ, C18(2), 100A, 250×4.6 analytical columns. The column was optimized and maintained at ambient temperature throughout analysis. Gradient system: Mobile phase phosphoric acid: methanol: water (A: 0.5:35:65 v/v/v and B: 0.5:50:50 v/v/v) shown below. It was filtered through 0.45 m nylon membrane filter and degassed in an ultrasonic bath prior to use with a flow rate of 1 mL/min. The injection volume was 20 µL; detection was set at 2 different wavelength 254 and 288 nm.

Time (min)	Mobile phase A (% v/v)	Mobile phase B (%v/v)
0-28	100 → 0	0 → 100
28-35	0	100
35-45	0 → 100	100 → 0
45-50	100	0

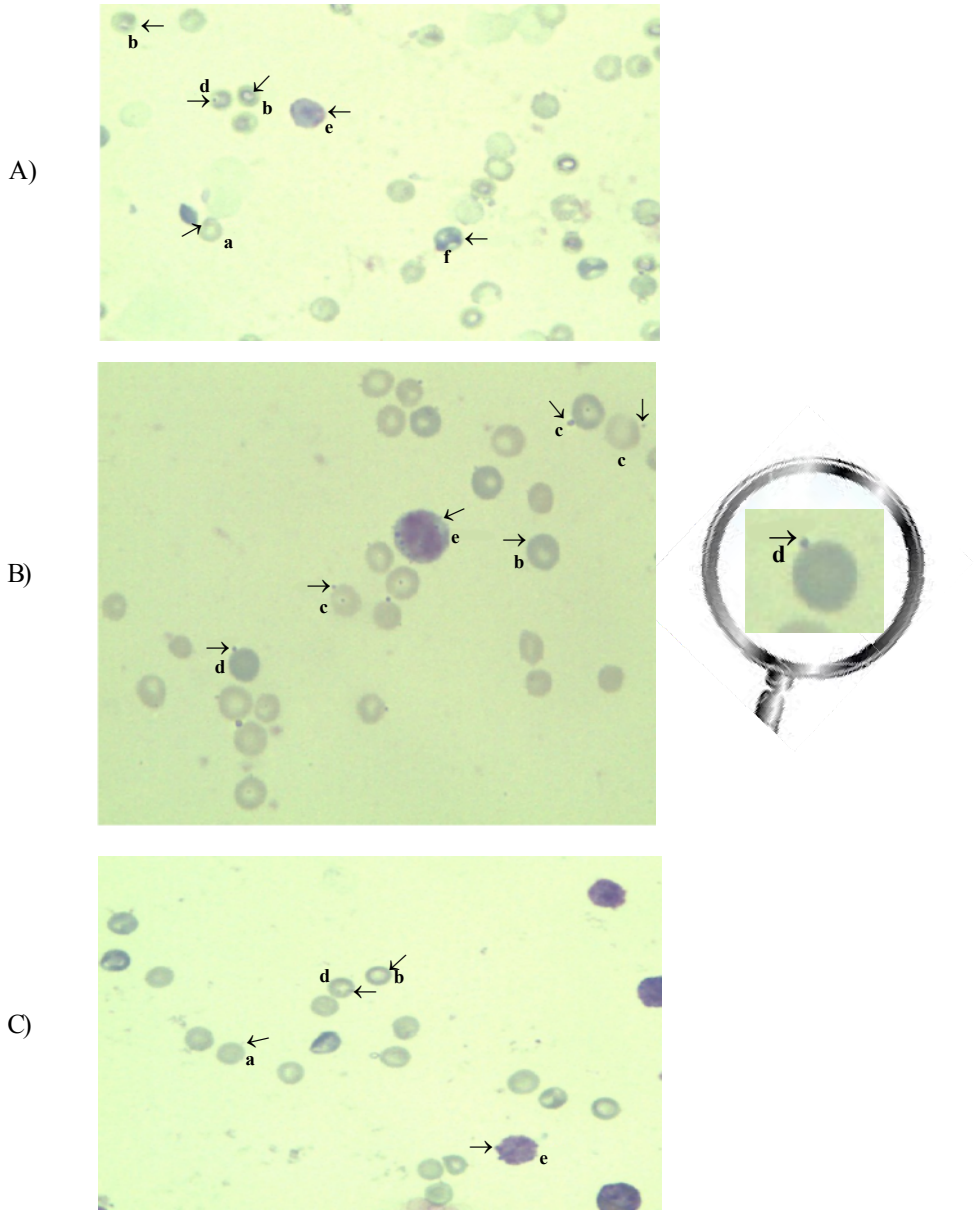

Figure-2: Effect of Linkus syrup and cyclophosphamide in rat bone marrow cells

Rat bone marrow (n=9-11) were collected from A) Control, B) Cyclophosphamide (20 mg/kg) and C) Linkus treated (1 and 5 g/kg) animals. Thin bone marrow smears slides were prepared and observed microscopically after staining with Giemsa (5%) at 100× magnification.

The arrows represent cell types: a) Normochromatic erythrocyte (NCE), b) Polychromatic erythrocyte (PCE), c) Normochromatic erythrocyte micronucleated (NCEM), d) Polychromatic erythrocytes micronucleated (PCEM), e) Blast cell and f) Neutrophils. The micunuclei are identifiable in B) in higher frequency as small round structures.

The present study demonstrated that administration of Linkus extract at 1 and 5 g/kg orally to Wistar rats for 3, 7 and 14 consecutive days did not induce any noticeable behavioral changes or mortality in mice or rats as compared to control group, therefore is non-toxic in both acute and sub-chronic toxicity tests. These results are in accordance with earlier studies (Nawaz *et al.,* 2014) further confirming that it is non-toxic in nature. It is worth mentioning that people suffering from respiratory ailments use Linkus for a recommended period of 3-7 days which is safe and so far no complaints have been received from its users.

It is well established that rat micronucleus assay is one of the most popular *in vivo* assay for the monitoring and assessment of genotoxicity of wide range of chemicals including pharmaceuticals, pesticides, petroleum products and water, food and air pollutants. Basically, when a bone marrow erythroblast develops into polychromatic erythrocytes, the main nucleus is extruded, any micronucleus that had been formed may remain behind in the otherwise anucleated cytoplasm. The visualization of such micronuclei is easier in these cells because they lack a main nucleus and hence provides a most unfailing tool for their detection. An increase in the frequency of micronucleated polychromatic erythrocytes in treated animals is an indicative that chromosomal damage has occurred favouring genotoxic action (Mavournin *et al.,* 1990, OECD 474, 1997).

Cyclophosphamide, is nitrogen mustard alkylating agent belongimg to oxazaphosphorine group and attaches the alkyl group to the guanine base of DNA, at the number 7 nitrogen atom of the imidazole ring. Thus interfering with DNA replication by forming intra-strand and inter-strand DNA crosslinks (Shulman-Roskes *et al.,* 1998). It is popularly used as positive control to induce micronuclei formation and in our study CP gavaged (20 mg/kg) to albino rats induced micronuclei formation in the bone marrow cells. It is reflected by a significant increase in micronuclei in both poly and normochromatic cells (PCEM and NCEM) as compared to control, thereby suggesting that CP act as genotoxic agent (Table 3). These results are inaccordance with earlier findings and also validates our experiments and the sensitivity of the animal strain used to the clastogenic agent as observed by earlier workers, (Sharma and Agarwal, 2015). On the contrary, in Linkus treated (1 and 5 g/kg) and control groups the number of micronuclei formed were similar and significantly lower than the cyclophosphamide treated group suggesting that polyherbal Linkus preparation has no mutagenic potential and does not induce micronucleus formation and hence it is non-genotoxic in nature. Furthermore, there were also differences in the number of micronucleus per cell, as a single micronucleus if any, was observed in few cells in control as well as in Linkus treated animals arising spontaneously. On the contrary, in case of CP treated animals the number of micronuclei per cell was about 2-3. The NCE demonstrated 2.7× greater micronuclei as compared to polychromatic erythrocytes per cells most likely due to its small cell size having smaller micronucleus which cannot be detected. Findings of other investigators regarding genotoxicity evaluation of individual medicinal plants (Table 4) present in Linkus preparation also supports our results. For example, the most predominat plant *A. vasica* reduced chromosomal aberrations and micronuclei formation in mice bone marrow cells in a dose dependent manner at 500 mg and 1000 mg/kg (Chincholkar *et al.,* 2012). Its leaf extract (800 mg /kg) fed for 7 consecutive days also provided protection in irradiated mice that induced undesirable changes in hematological and biochemical parameters

Table 3: Micronuclei Formation in Rats Poly- and Normo-chromatic Erythrocytes in the Presence and Absence of Cyclophosphamide and Linkus Cough Syrup

S.No.	Groups	PCE			NCE			PCE/NCE	MN (%)	
		MN(-)	MN(+)	Total	MN(-)	MN(+)	Total		PCE	NCE
1.	Control (Water)	2413±60.5	1.3±0.3	2414	288.2±12.6	1.1±0.3	289	8.3	0.05 ±0.01	0.35±0.08
2.	CP (20 mg/kg)	2773±175.9	3.8±1.7	2777	375.5±61.5	10.3±1.4	386	7.2	0.14 ±0.07	2.8±0.2
3.	Linkus 1 g/kg (14 days)	2503±105.2	1.8 ±0.4	2505	386±26.5	1.3±0.4	387	6.5	0.06±0.02	0.3 ±0.09
	Linkus 5 g/kg (14 days)	2470±59.3	1.5±0.3	2471	393.5±30	1.1±0.5	395	6.3	0.06 ±0.01	0.3±0.09

(CP) = Cyclophosphamide; (PCE) = Polyochromatic erythrocytes and (NCE) = normochromatic erythrocytes
Without micronucleus MN (–) and with micronucleus MN (+)
Total PCE and NCE: MN (–) plus MN (+) cells. Range for PCE = 2414 to 2777 and NCE = 289 to 395.
The values presented are mean±SEM of PCE and NCE with and without micronuclei (n=9-12).

(Kumar *et al.,* 2005). However, it was reported to be mito-depressive in the plant system, as in *Allium cepa* root meseristem (Borooah, 2011) indicating that, although *A. vasica* has beneficial effects as a medicinal herb, and is non-genotoxic but it may damage cells emphasizing if used as one of the component in any herbal formulation, the concentration and duration of its use must be taken into account.

The second most predominant plant in Linkus preparation is *G. glabra* root and its extract did not induce any chromosomal aberrations (Sharma and Agrawal, 2015). Galangin, a member of the flavonol class of flavonoid, present in high concentrations in it and in other medicinal plants (e.g. *A. galanga* L.) and propolis, a natural bee hive product suppressed the genotoxin actions and may be a promising candidate for cancer chemoprevention (Heo *et al.,* 2001). Likewise, *G. glabra* L. leaves (Siracusa *et al.,* 2011) and hydromethanolic root extracts (Sharma and Agrawal, 2015) provided significant protection against cyclophosphamide induced-chromosomal aberrations in the mice bone marrow and consequently appeared as anti-clastogenic and anti-mutagenic which was linked with polyphenols, namely flavonoids and dihydrostilbenes.

Moreover, PCE:NCE ratio also provides an index of cytotoxicity with DNA damages leading to cell death or apoptosis (Ouanes *et al.,* 2003). In our study, the PCE:NCE ratio in the control (8.3) was significantly higher than Linkus treated group at both doses i.e. 1 and 5 g /kg (Table 4) indicating that Linkus with lower PCE/NCE ratio is more likely to be cytotoxic possibly by interrupting the mitotic cell division and favouring its mito-depressive actions. This property could be associated with other plants present in it. As the water soluble allelopathic infusions from *H. officinalis* L. exerted mito-depressive effects on the rate of cell division in *Cucumis sativus* L. and *Triticuma estivum* L. while genotoxic in *Allium cepa* root tip cells (Dragoeva *et al.,* 2010). Also *V. odorata* extract (5-50%) containing bioactive principle, violin and probably methyl salicylic ester elicited a dose dependent clastogenic effect in both mitotic (root tip) and meiotic (buds) cells of *Vicia faba* implying that the plant is cytotoxic at higher doses again emphasizing the importance of safe concentrations of herbal products to be used (Asthana and Kumar, 2014).

Although, the micronuclei assay is very useful as a short-term screening method but it is difficult to evaluate the safety of chemical substances based on single short term test system. It is also recommended in the guidelines for genotoxicity studies of chemical substance that a combination of *in vitro* bacterial reverse mutation assay (Ames test), *in vitro* mammalian chromosomal abnormal test along with *in vivo* rodent micronucleus assay could be used in series (Maron and Ames, 1983). The *in vivo* micronucleus assay in the bone marrow cells is used to identify chromosome aberrations, thus the target cells are effectively exposed to the chemicals or test substances (Sato and Tomitab, 2001). However, the bone marrow micronucleus assay may not be suitable for clastogenic compounds,which are reactive to other cellular micronucleus and have barrier in reaching bone marrow cells (Sato and Tomitab, 2001).One has to bear in mind that the compounds administered orally,intraperitoneally or intravenously may be inactivated before it reaches the target bone marrow cells thus, a micronucleus assay using other organs or tissue should also be conducted to reach a definite conclusion.Nevertheless, findings of the present investigation led us to conclude that Linkus (5 g/kg) administered orally to rodents in acute and sub-chronic toxicity test

Table 4: Toxicity and Genotoxicity of Some Medicinal Plants Present Linkus Syrup

S.No.	Medicinal plants		Model and doses	Toxicity/genotoxicity testing endpoints	Activities	References
	Botanical name	Common name				
1.	*Adhatoda vasica* Nees	Bansa	Swiss albino mice (500 & 1000 mg/kg)	Reduction in radiation-induced chromosomal aberrations and micronuclei	Radioprotective	Chincholkar, *et al.,* (2012)
2.	*Glycyrrhiza glabra* L.	Mulethi	Swiss albino mice (300, 450 & 600 mg/kg)	Protection against cyclophosphoamide-induced chromosomal aberration in bone marrow cells	Antimutagenic	Sharma *et al.,* (2015)
3.	*Piper longum*	Filfil daraz	Swiss albino Mice Acute 0.5, 1.0 and 3 g/kg, chronic 100 mg/kg	No toxic effects in acute or chronic test	Non-toxic	Shah *et al.,* (1998)
4.	*Althea officinalis* L.	Khatmi	Albino Mice (2.5 kg & 5 g/kg)	No changes in biochemical parameters.	Non-toxic	Soleimany *et al.,* (2015)
5.	*Hyssopus officinalis* L.	Zufa	*Allium cepa* (17.5g/L, 52.5g/L)	Mitodepressive and genotoxic effect in root tip cells	Allelopathic activity	Dragoeva *et al.,* (2010)
6.	*Alpinia galanga* L.	Khulanjan	Swiss albino mice (0.1-100 mg/kg)	Galangin: Anti-oxidant, modulate enzyme activities, and suppresses genotoxic effects	Chemo-preventive agent	Heo *et al.,* (2001)

for 14 days is non-toxic. Likewise, in *in vivo* rat bone marrow test, Linkus (5 g/kg) treatment for 14 days did not induce micronuclei formation in rat bone marrow test thereby favouring that it is non-genotoxic, hence supporting its safe usage in humans.

Author's Contribution

Saira Bano, Sayedul Haque, Aqib Zahoor and Rabia Ali performed the experiments Ahsana Dar Farooq supervised the entire research project.

Conflict of Interest

The authors declare that there are no conflicts of interest.

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Varicocele: Prevalence, Incidence and Its Related Testicular Atrophy

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Abstract

Varicocele is characterized by abnormal tortuosity and dilatation of the pampiniform plexus veins within the spermatic cord and it is one of the causes related to male infertility. The aim of our study was to determine the prevalence of varicocele, its site incidence of secondary infertility due to varicocele and related testicular atrophy in adults. The study was conducted on 175 patients presenting with infertility and other problems. Of 175 patients, 75 were diagnosed with varicocele with prevalence of varicocele to be 22.85% and the incidence of 45.0% in adults. The complaint of infertility in the population with varicocele was 72.7%, atrophy of testis was 22.5% and it was associated with severity of varicocele. We conclude that early diagnosis of varicocele is important to prevent future infertility.

Keywords

Vericocele, Pampiniform, Prevalence, Atrophy.

1. INTRODUCTION

A Varicocele is an abnormal tortuosity and dilatation of the pampiniform plexus within the spermatic cord that results from valvular incompetence of the spermatic vein. The prevalence of varicocele is approximately 15-20% in the general population and 30-40% in infertile males and upto 80% in cases of secondary infertility (Clarke, 1966; Johnson *et al.,* 1970; Jarow, 2001). It rarely appears before age of 10 years and it tends to persists throughout life, if left untreated. The definitive etiology of varicocele is not well known, but it's increased frequency of presentation on the left side has led to the discussion of several theories. These include an increased length of left spermatic vein, it's right-angle entry into the higher pressure left renal vein, an increased absence of valves in the left spermatic vein compared with the right and the possibility of the "nutcracker" phenomenon with compression of left renal vein between the aorta and superior mesenteric artery (Saypol, 1981; Coolsaet, 1980). Varicocele are almost always larger and more common on the left side, upto 50% of cases have bilateral varicoceles (Abdulmaaboud *et al.,* 1998).Varicocele are detected and graded by physical examination (Dubin *et al.,* 1970; Amelar *et al.,* 1987; Dubin *et al.,* 1971). Early detection is important because varicocele occasionally may cause infertility and rarely it's a sign of intra-abdominal cancer (Clarke, 1966; Dubin *et al.,* 1971; Turner, 1983).

The aim of the present study was to

determine the prevalence, site of varicocele, incidence of secondary infertility due to varicocele and varicocele related testicular atrophy in adults.

2. MATERIALS AND METHODS

Study was conducted on 175 patients presenting with infertility and other symptoms in Department of Jarahat (Surgery), Ajmal Khan Tibbya College & Hospital, AMU, Aligarh.

Patients of age group 15-40 years attending surgery OPD were screened and the patients with coexisting morbid conditions like diabetes, heart disease, malignancy, haematologic disorders, history of drug abuse or chronic debilitating diseases were excluded.

Patients selected in the study were thoroughly examined. The volume, position and consistency of the testes and epididymis were examined, and each spermatic cord was palpated in the standing position and during the Valsalva maneuver. Findings were graded according to the system of Dubin *et al.,* 1970 and Amelar *et al.,* 1987 as follows.

Grade I: Varicocele palpable only during Valsalva maneuver in upright position.

Grade II: Varicocele palpable in upright position without the aid of Valsalva maneuver.

Grade III: Varicocele is both palpable and visible through the skin of scrotum without the aid of Valsalva maneuver in the upright position.

Scrotal ultrasound was done to evaluate the testicular size and testicular atrophy was defined as any testis with a volume of <15 ml or a testis 25% or smaller than it's contralateral mate.

3. RESULTS AND DISCUSSION

Out of 175 patients suffering from different disease conditions, 75 were diagnosed with genital problems or infertility. After investigations among these 75 patients 40 were diagnosed with varicocele and 35 patients were found to have diseases without varicocele. We found that the incidence of varicocele was 22.85%, among 40 patients 29 patients were unmarried and 11 were married. The study showed that varicocele associated with infertility in 72.7% of the population

Maximum number (45%) of cases were found to be in age group of 21-25. 27.5% cases are in age group of 15-20 years and 22.5% in age group of 26-30 years. The diagnosis in age group (15-20) was highlited by chance with mild discomfort or pain and swelling (Table 1). Table 1 also shows distribution of varicocele in married and unmarried population. In age group of 15-20 years 01 patient was married as well as infertile. In 21-25 years of age group 05 (29.41%) were married and of these 05, three were infertile. In 26-30 years of age group, 8 patients were married and of them 3 were infertile. In 31-35 years of age group, only one patient reported, who was married and infertile. In 36-40 years of age group, 01 patient reported and he was fertile. The earliest description of varicocele dates back to the first century A.D., The famous Roman Physician Celsus (30 B.C.-45 or 50 A.D.) discussed testicular atrophy with swollen scrotal vein in his book La Medicina (Kaufman *et al.,* 1987). At present varicocele is recognized as the leading cause of male infertility. The prevalence of varicocele has been studied extensively and the results are complex. It is present in approximately 15% of all population. In contrast, 35% of men with primary infertility and 70% of men with secondary infertility present varicocele (Witt *et al.,* 1993). In one of the large study group the prevalence was 11.7% in general population and 25.4% in infertile male population (Madgar *et al.,* 1995; Nieschalg *et al.,* 1995). Akbay *et al.,* (2000) detected varicocele in 293 (7.2%) of 4052 patients. In Pfeiffer *et al.,* (2006) performed a

Table 1: Distribution of Varicocele in Different Age Groups

Age group in years	Patients		Marital Status		No. of Patients
	No. of Patients	%	Married	Unmarried	
15-20	11	27.5	01	10	01
21-25	18	45.0	03	15	03
26-30	09	22.5	05	04	03
31-35	01	2.5	01	00	01
36-40	01	2.5	01	00	00
Total	40	100	11	29	08 (72.7%)

doppler based study on the prevalence of varicocele in 2008 (42.7%). They concluded that this condition is common in adults. Mickenicius and Bosas (2002), studied one hundred patients and they found varicocele in 24% of all patients. In this research study varicocele was prevalent in 22.85% cases with similarity to previous studies.

It has been reviewed that limited emphasis has been placed on the incidence of varicocele in adolescents. 10-15% varicocele incidence is reported in adolescents (Ostler, 1971). This research study reported 45.0% incidence of varicocele in adolescents (Table 1). These patients require a close follow-up and treatment to prevent the future fertility problems. The peak age of diagnosis is 21-25 years, as observed in the study.

Table 2 shows the distribution of disease in patients without varicocele. Out of 75 patients, 35 patients were not suffering from varicocele. Primary infertility was noted in 20% of patients, 06 (17.14%) of them were reported with the complaint of having primary infertility, while 04 patients (11.42%) were having undescended testis. Atrophy of testis was reported in 04 (11.42%) patients, 10 (28.57%) were having hydrocele, 9 (25.71%) patients suffered from epididymorchitis. One 01 case (2.85%) of arteriovenous malformation, and 01 case (2.85%) of urinary tract infection was also observed. It has been observed that 35 patients examined without complain of varicocele, 07 (20.0%) patients were found infertile.

Infertility is one of the important issues of human societies. The average prevalence of infertility in different societies is 8-12% (WHO, 2014) with numerous curative factors. Many studies have been conducted dealing with male infertility and factors affecting it (Sohrabvand *et al.,* 2015). Varicocele associated with infertility has been recognized globally and is found in 75%-81% cases with secondary infertility (Miyaoka *et al.,* 2012), in this study, 72.7% secondarily infertile participants were affected by varicocele.

Table 2: Distribution of Disease in Patients without Varicocele

Diagnosis	No. of patients	No. of Patients with infertility
Primary Infertility	06	06
Undescended testis	04	–
Atrophy of testis	04	–
Hydrocele	10	–
Epididymorchitis	09	–
Arteriovenous Malformation	01	01
Urinary tract infection	01	–
Total	35	07 (20%)

Table 3 shows (70%) cases with unilateral and (30%) cases of bilateral varicocele. Considering unilateral cases (92.8%) will left unilateral while only 7.2% will right unilateral annomalies. It is still a dilimina as why vericocele occurs. There are many theories attributing them to the lack or incompetence of valves in the internal spermatic vein (Brown, 1975), the junction between the spermatic vein and left renal vein (Herzinger, 1981), regonadal bypass (Mali et al., 1986), decrease of the activity of the cremaster with the spermatic cord (Shafik, 1973), compression of the left renal vein between the aorta and the inferior mesenteric artery (El-Sadr *et al.,* 1950), abdominal tumor (Anderson, 1968) and renal tumor on either side (Weinerth, 1986).

It is also not clear that why varicocele is more common on the left side, although this has been attributed to the pressure caused by descending or sigmoid colon (Mali et al., 1986). Fifteen percent of males have varicocele, which is predominantly left sided (approximately 85-90%), although bilateral varicocele has been found in upto 30% of cases (Comhaire, et al., 1998). In our study the predominance of left sided varicocele was 92.8% with 30% bilateral cases of varcocele. The reason behind this phenomena may be that the left testicular vein drains into the left renal vein and may suffer compression under the superior mesenteric artery and abdominal aorta. Moreover, the drainage of the left testicular vein the left renal vein, that too almost at right angle may predispose the condition.

Table 4 demonstrates that the atrophy is associated with severity of varicocele. In the patients with grade I varicocele 8.33% of the patients developed atrophy of the testis, 50% of the population with atrophy of the testis was found to have grade II varicocele, while atrophy was observed in 38.46% of the patients with grade III varicocele.

The importance of varicocele lies in its

common presentation in general male population and in infertility clinics. Celsus in the first century A.D. noted testicular atrophy on the side of varicocele (Sherins *et al.,* 1986).Various research studies demonstrated varicocele as a cause of male infertility and testicular atrophy and It is widely accepted that varicocele can cause progressive damage to the affected testis, leading to testicular atrophy and reduced fertility (Gorelick *et al.,* 1993; Kass *eet al.,* 2001; Greenspan *et al.,* 2004; Daitch *et al.,* 2001; World Health Organization, 1992).We also noted the atrophy of testis in our study in 22.5% of cases and it's association with severity of varicocele.

4. CONCLUSION

It has been concluded that prevalence of varicocele was found to be in 22.85% population, and the incidence of varicocele in 45.0% of adolescents (21-25 years of age group). Our findings supported the previous studies in respect to the predominance of left sided varicocele. In this study 72.7% secondarily infertile participants were affected by varicocele and 22.5% of cases with testicular atrophy. The testicular atrophy was found to be associated with severity of varicocele. Therefore, it can be concluded that the peak age of diagnosis of varicocele is 21-25 years and patients of varicocele are at increased risk of arrested testicular growth leading towards testicular atrophy. Thus early diagnosis is important to prevent future infertility.

Conflict of Interest

There is no conflict of interest.

Table 3: Distribution of the Patients According to the Effected Side

Patients with Unilateral varicocele		Patients with Bilateral varicocele
28 (70%)		12 (30)%
Right	**Left**	
02 (7.2%)	26 (92.8%)	

Table 4: Grading of Varicocele Associated with Testicular Atrophy

Grade	No Atrophy	Atrophy	Percentage
I	12	01	8.33
II	06	03	50.00
III	13	05	38.46
Total (40)	31	09 (22.5%)	

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Hyperlipidemia in Unani Perspective – A Review

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Abstract

The hyperlipidemia is a life style disorder and has affected mankind since antiquity. Detailed work has been carried out to reduce the burden of this disease and many drugs have been proposed in different systems of medicine to overcome the situation and to minimize complications. Unani system of medicine also has significant contribution in this regard and a number of studies have been carried out and many studies are going on for the same. Here, it is aimed to explore the possible concept of hyperlipidemia in Unani system of medicine. A disease *Siman-i-mufrat* is discussed in ancient Unani literature, which has many similarities with hyperlipidemia. Hyperlipidemia is a major health problem in entire world and responsible for the development of atherosclerosis and hence in coronary artery disease and many fatal conditions. The Unani system can provide a valuable contribution towards reduction of the disease development and help to decrease its complications. In Unani system of medicine, the concept of fat or oily substance in blood is present and was narrated as *Dasoomat-fid-dam*.

Keywords

Hyperlipidemia, *Siman-e-mufrat, Dasoomat-fid-dam*.

1. INTRODUCTION

The term hyperlipidemia indicated abnormal increase of lipids or lipoproteins in blood. There is no description of this disease found in ancient Unani literature. The possible reason behind is absence of techniques required for diagnosis of the disease.

Unani scholars were also aware about the presence of fat in circulating blood and described it as *Dasoomat-fid-dam*. Abu Sahal Masihi mentioned in his famous book, *Kitab-ul-Miaát* about the presence of fat in blood and emphasized that this fat mixed blood is responsible for the development of fat which is found in different parts of the body (Masihi, 1963). In the text, *Moalajat-e-Nafeesi,* the author gave hints regarding fat in circulation as he described that the lighter part of fat travels with circulating blood and reaches to the organs and provides nutrition to that organ (Nafees bin Auz bin Jamaluddin, 1910).

It is very much true that there is no description of any disease by the name hyperlipidemia in Unani literature but it has been understood by the above discussion that Unani scholars were well aware about the presence of fat in circulating blood. So, the disease hyperlipidemia can be named as *Fart-e-tadassum-fid-dam* or *Kasrat-e-dasoomat-fid-dam*.

Siman-e-mufrat is the only disease found in Unani literature, which has resemblance with hyperlipidemia in its clinical picture, its aetiology and obviously its complications or consequences. Although, the pathophysiology of hyperlipidemia and obesity is not similar to each other but because of other similarities, it is fair to compare these both conditions. It is also true that hyperlipidemia is very much common among obese patients and it is liable to say that hyperlipidemia has somehow a close relationship with obesity and possibly discussed by the Unani philosophers as *Siman-e-mufrat*.

Hippocrates (460 B.C.), known as the father of medicine, described the disease, *Siman-e-mufrat,* in *Fusool-e-Buqratia* and it is a vivid description. Galan is a very familiar name in the Unani world, wrote *Fusool-e-Buqrat-ma-Talkhees-e-Jalinoos* in which he wrote in reference to Hippocrates that an obese person lives a shorter life than a lean and thin person (Jalinoos, 1903). Thus, Hippocrates was the first Unani physician who discussed *Siman-e-mufrat* as a disease. Rofas suggested that an obese person has less tolerance of fasting, involve in digestive problems, less capacity for hard works and obesity leads to fatal diseases because of increased amount of *Khilt-e-balgham* and less amount of *Khoon-e-saleh.* In females, obesity leads to abortion or unhealthy child birth. Rofas also quoted that purgatives have fatal effect on these peoples. Some dangerous diseases mentioned by him as a complication of obesity are epilepsy, paralysis, syncope and high grade fevers (Al-Qamari, 1255 H.). Galen (129-200 A.D.) gave a valuable description about obesity and its management with the help of *Istafragh* mentioning that in obese person, major blood vessels are prone to rupture and sudden death may occur (Jalinoos, 1903). Rabban Tabri (810 A.D.), Unani physician mentioned the causes of obesity like intake of heavy meals, prolong sleeping and prolong rest; clinical features like nausea, decreased semen; complications like paraplegia and facial paralysis and sometimes sudden death, in *Firdaus-al-Hikmat* (Tabri, 2010). Zakariya Razi (885-925 A.D.) was very much popular because of book titled as *Al-Havi Fit-Tib* describing sixth volume, obesity, including its etiology, dietary factors, clinical presentation, types, management and complications with greater emphasis on diet in the management of the disease and also mentioned the role of diuretics and vinegar in reduction of body fat. According to Razi, alcohol if taken in small quantity, reduces fat. Majoosi described the types of fat and its distribution in the human body mechanism of fat synthesis. In the context of disease, obesity is a fatal disease if primarily occurs. Due to excessive fat the blood vessels are compressed and hence become narrow due to which infusion of *Hararat-e-gharizi* become difficult and it may leads to death. In their life they suffer from diseases like paralysis, sterility, dyspnoea and stroke. Due to less oxygen in their blood, heavy exercise may prove fatal (Majoosi, 2010).

Sheikh Ibn Sina quoted that due to excessive fat, narrowing of blood vessels occurs which in turn reduces the oxygen supply in blood reduced movement; *Hararat-e-gharizi* diminishes and disturbs temperament. Blood vessels are prone to rupture and may lead to death. Dyspnoea and palpitation are the common features. Excessive fatty peoples are also prone to early death especially if obesity occurs in early age. Complications described are paralysis, shock, purgation, syncope and many types of fever. They have less tolerance of thirst and hunger, temperament becomes cold, less blood and more phlegm. Sterility is an important complication and quantity of semen is also

reduced, while in females abortion is common. Fasad and Ishaal are dangerous in these peoples. Decrease in diet, empty stomach hot bath, heavy exercise and some life style modifications are described by Sheikh for the management of obesity (Ibn Sina, 2010). Ismail Jurjani (1140-1236 A.D.) in his literature named *Zakheera Khwarzam Shahi* quoted that obesity has a numerous complications. The author suggestion about obesity is that its causes narrowing of vessels, early death, paralysis, shock, syncope, decreased activity, purgation, sterility and impotence in males, abortions in females, decreased response to drugs which leads to delay in treatment of diseases (Jurjani, 2010). Ibn Nafees (1207-1288) was the first Unani physician to provide information about the relation of obesity with cardiovascular disorders, cerebrovascular as well as respiratory diseases (Ibn Nafees, 1984).

2. *MAHIYAT-E-MARZ* (PATHOLOGY)

In Unani literature ancient Unani scholars described the *Mahiyat-e-marz of Siman-e-mufrat*. It was explained that person having more fat in the body is at risk of rupture of blood vessels because his vessels are compressed due to excess of fat and especially if obesity develops in early stages of life, then the blood vessels become narrow. These narrow and compressed vessels are not able to fulfill the demand of *Rooh-e-haiwani* and causes diminution of *Hararat-e-gharizi* (Ibn Sina, 2010; Jurjani, 2010; Nafees Kirmani, 2010). The excess of fat leads to improper digestion and so disruption of *mizaj*, which become *barid* and *Shiddat-e-burudat* may cause death (Jurjani, 2010; Ibn Rushd, 1987). The symptoms arise due to narrow blood vessels and *Burudat-e-mizaj*, both effect different organs of body like liver, lungs, heart, organs of reproduction and kidneys. (Ibn Sina, 2010; Jurjani, 2010; Jeelani, 2010). Effect of drugs becomes delayed due to narrow blood vessels as it reaches to the target after mixing with blood (Ibn Sina, 2010). Hence, it is difficult to say that the features of the disease can't be defined with the help of this *Mahiyat-e-marzi*.

3. *ASBAB* (CAUSES)

Various causes for *Siman-e-mufrat* are described in Unani literature, which are as follows:

1) Maurusi (hereditary) in some families (Jeelani, 2010).
2) *Khilqi* (congenital) (Al-Qamari, 1255 H.).
3) After menopause, it is common in females (Jeelani, 2010).
4) *Kasrat-e-ghiza* especially fatty and oily substances and those substances which are rich in sugar and starch (Tabri, 2010; Jeelani, 2010).
5). Use of butter and excess of milk especially of buffalo (Jeelani, 2010).
7) Use of alcohol (Al-Qamari, 1255; Jeelani, 2010).
8) Sedentary life style (excess of joy, more rest, lack of exercise etc.) (Tabri, 2010).
9) Excessive sleeping (Tabri, 2010).
10) Conversion of blood into fat (Ibn Rushd, 1987).

Many of these above mentioned causes of disease *Siman-e-mufrat,* resembles with the causes described in modern system of medicine for the disease hyperlipidemia.

4. *ALAMAAT WA AWARIZAT* (SYMPTOMS AND COMPLICATIONS)

In Unani text, the clinical features and

complications are discussed side by side. These are as follows:

i) *Taqleel-e-harkat* (Jurjani, 2010; Ibn Nafees, 1984).
ii) *Khafkhan* (Ibn Sina, 2010; Jurjani, 2010)
iii) *Zeeq-un-nafas* (Ibn Sina, 2010; Jurjani, 2010).
iv) *Hummiyat* (Al-Qamari, 1255 H.; Ibn Sina, 2010; Jeelani, 2010).
v) *Sakta* (Ibn Sina, 2010; Jurjani, 2010; Nafees Kirmani, 2010).
vi) *Ghashi* (Al-Qamari, 1255 H.; Ibn Sina, 2010; Jurjani, 2010; Nafees Kirmani, 2010).
vii) Susceptibility to vomiting and loose motions (Tabri, 2010; Ibn Sina, 2010).
viii) Impotence and sterility in males (Ibn Sina, 2010; Jurjani, 2010; Nafees Kirmani, 2010).
ix) Sterility and abortions in females (Al-Qamari, 1255 H.; Ibn Sina, 2010; Jurjani, 2010; Nafees Kirmani, 2010).
x) Lack of libido (Tabri, 2010; Ibn Sina, 2010; Jurjani, 2010).
xi) Hemorrhages (Jurjani, 2010).
xii) *Falij wa Laqwa* (Al-Qamari, 1255; Ibn Sina, 2010; Jurjani, 2010; Nafees Kirmani, 2010).
xiii) Epilepsy (Al-Qamari, 1255 H.).
xiv) Increased susceptibility to diseases and delayed response of medications (Jurjani, 2010; Nafees Kirmani, 2010).
xv) Sudden death (Tabri, 2010; Jurjani, 2010; Nafees Kirmani, 2010).

5. *USOOL-E-ILAJ* (PRINCIPLE OF MANAGEMENT)

In Unani system of medicine, *Usool-e-ilaaj* has a nuclear value in the treatment of any disease. The following principles should be followed for the management of *Siman-e-mufrat*.

1. *Taqleel-e-ghiza* or a diet which is bulky but less nutritive (Ibn Sina, 2010).
2. *Hamam-e-yabis qabl-az-taám* (Ibn Sina, 2010; Nafees Kirmani, 2010; Arzani, 2010).
3. *Riyazat-e-shadeeda* (Tabri, 2010; Jurjani, 2010; Nafees Kirmani, 2010; Jeelani, 2010).
4. *Mehnat-o-mashaqqat* (Tabri, 2010; Arzani, 2010).
5. *Roghaniyat-e-muhallila wa musakh-khina* (Ibn Sina, 2010; Nafees Kirmani, 2010; Arzani, 2010).
6. *Mushilat, muarriqat and mudirrat-e-bol* drugs (Nafees Kirmani, 2010; Arzani, 2010; Jeelani, 2010).
7. *Har-yabis wa muhallil* drugs (Nafees Kirmani, 2010).
8. Reduction in sleep (Al-Qamari, 1255; Arzani, 2010).
9. Use of hard bedding (Ibn Sina, 2010).
10. Use of vinegar (Ibn Sina, 2010; Jurjani, 2010).
11. Not to use sweet and oily substances (Jeelani, 2010).
12. Brisk walk daily (Jeelani, 2010).

So, the *Usool-e-ilaaj* comprise of three types of treatment i.e. *Ilaaj-bil-ghiza, Ilaaj-bil-tadbeer* and *Ilaaj-bil-dawa*.

A number of drugs were used for the treatment of *Siman-e-mufrat* although it is very difficult to treat. The single drugs described in ancient Unani literature are *Biladur* (Tabri, 2010), *Muqil* (Tabri, 2010), *Nankhah* (Arzani, 2010), *Badyan* (Arzani, 2010; Jeelani, 2010), *Suddab* (Arzani, 2010; Jeelani, 2010), *Zeera kirmani* (Razi, 1999; Arzani, 2010; Jeelani, 2010), *Luk maghsul* (Arzani, 2010; Jeelani,

2010), *Marzanjosh* (Arzani, 2010; Jeelani, 2010), *Boora-e-armani* (Arzani, 2010; Jeelani, 2010), *Sandrus* (Arzani, 2010), *Zaaj* (Arzani, 2010), *Zarawand* (Arzani, 2010), *Juntiyana* (Arzani, 2010), *Sirka* (Arzani, 2010), *Raai* (Jurjani, 2010), *Lehsun* (Jurjani, 2010) etc.

Compound drugs that treat *Siman-e-mufrat* in unani medicine are *Roghan-e-shibbat* (Nafees Kirmani, 2010; Arzani, 2010), *Roghan-e-qust* (Nafees Kirmani, 2010; Arzani, 2010) for local application and *Itrifal sagheer* (Ibn Sina, 2010; Jurjani, 2010), *Tiryaq-e-kabir* (Jurjani, 2010), *Namak-e-afaee* (Jurjani, 2010), *Jawarish kamooni* (Jurjani, 2010), *Majoon falafali* (Jurjani, 2010; Nafees Kirmani, 2010), *Anqarooya* (Jurjani, 2010), *Asanasiya* (Jurjani, 2010), *Amroosiya* (Jurjani, 2010), *Dawa-e-luk* (Nafees Kirmani, 2010; Arzani, 2010) for internal use.

So, an effort has been made to describe hyperlipidemia in the light of Unani literature which may further help in the contribution of Unani medicine towards public health.

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Spasmolytic Activity of the Aqueous Extract of Leaves of *Coccinia grandis* L. in Rabbit Smooth Muscles

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Abstract

This study was aimed to provide pharmacological basis for the medicinal use of *Coccinia grandis* L. (Cucurbitaceae) leaf aqueous extract in indigestion and constipation using the *in vitro* assay. The portion of rabbit jejunum was mounted in an organ bath, filled with Tyrode's Ringer solution and spontaneous movements of intestine were recorded on oscillograph and kymograph using isotonic transducer before and after applying drug samples and aqueous leaf extract. This study showed that the *C. grandis* L. possesses laxative activities along with spasmodic effect in the isolated tissues, mediated partially through activation of muscarinic receptors; thus, this study provides a rationale for the medicinal use of *C. grandis* L. leaves in indigestion and constipation. The results showed that the aqueous extract of *C. grandis* L. leaves has a significant spasmodic activity which supports its use in traditional herbal medicine practice. The extract produced an increase in intestinal transit comparable to normal contraction; number of contraction also increased in the presence of extract. Hence, the result indicated a remarkable increasing effect on the movement of intestinal tissues of rabbit in comparison to control.

Keywords

Spasmolytic activity, *Coccinia grandis*, Jejunum, Tyrode's Ringer solution.

1. INTRODUCTION

Constipation, diarrhoea, colic and cramps are triggering 70% of the world's population. (Quyang and Chin, 2004). Constipation is the most common gastrointestinal complaint all over the world resulting in over two million reported cases annually (Rawat *et al.,* 2012). About 12-19% of Americans (Eoff, 2008) and 14% of Asians (Cheng *et al.,* 2003) suffer from constipation, with symptoms varying by geographic location (Tally *et al.,* 1993). It can disturb persons of any age. It starts just after the birth at the rate of 3%, go above 50% at the age 50, and then reaches above 90% at the age of 80. It manifest from enlarged haemorrhoids to colon cancer, and the lot in between. Some people who suffer constipation feel pain during bowel movement often experiencing with straining, bloating, swelling, uncomforting and the feeling of a full bowel. Constipation happens if the colon absorbs excessive water or if the colon's muscle contractions become slow or sluggish which retards stool movement over the colon. As a result, stools become hard and dry. Constipation is diagnosed if no bowel movement occurs for three or more days and if this irregularity persists for more than six months (Hasler and Owyang, 1995).

Indeed, numerous OTC laxatives comprise of herbal components. Laxative herbs mostly contain anthraquinones, or constituents which

have stimulanting effect on the intestines. These laxatives exerts its effect by drawing in liquid to the colon and increasing peristalsis.

Coccinia grandis L. (Cucurbitaceae) is distributed in tropical Asia, Africa and is commonly found in Pakistan, India and Srilanka (Cooke, 1903; Sastri, 1950). It is a climber and trailer (Nasir and Ali, 1973). The fruit of *C. grandis* L. is used as vegetable when green and eaten fresh when ripens into bright scarlet colour (Sastri, 1950). Every part of this plant is valuable in medicine and various preparations have been mentioned in indigenous system of medicine for skin diseases, bronchial catarrh, bronchitis. In Unani system of medicine recommended for ring worm, psoriasis, small pox, and other itchy skin eruptions and ulcers (Perry, 1980; Behl *et al.,* 1993). Oil of this plant is used as an injection into chronic sinuses. The plant is also useful in decoction for gonnorhoeae, diabetes and also beneficial in dropsical condition, pyelitis, cystitis, strangury, snake bite, urinary gravel and calculi (Nadkarni, 1976; Jayaweera, 1980). It is also worthwhile to induce perspiration in fever and cures sores in the tongue (*Dictionary of Indian Medicinal Plants,* 1992). It has antilithic, hypolipidimic, antimutagenic, laxative and hypoglycemic activities (Chopra and Bose, 1925; Gupta, 1963; Brahmachari and Augusti, 1963; Mukherjee *et al.*, 1972; Presanna *et al.,* 1997; Nahar *et al.*, 1998; Kusamran *et al.*, 1998). The present study was undertaken to evaluate spasmolytic activity of *Coccinia grandis* L. extract.

2. MATERIAL AND METHODS

2.1. *Plant Material and Preparation of Extract*

Coccinia grandis L., leaves were procured from Lasbela (Balochistan, Pakistan). It was authenticated by Prof. Dr. Ghazala H. Rizwani (Meritorious), Department of Pharmacognosy, Faculty of Pharmacy, University of Karachi. It was grinded and then soaked in boiled water for about 1 hour and rotated at 40°C on rotary evaporator (Buchi Rotavapor R-200) and then filtered and evaporated by means of lyophilizer (Eyela freeze dryer Fd-1) into dry crude extract.

2.2. *Animals*

Local breed rabbits (weighing 1-1.5 kg) of either sex were used for the experiment and housed under a controlled environment (23-25°C). Animals were purchased from the animal house of University of Karachi. Rabbits were kept in plastic cages (47 × 34 × 18) and fasted for 24 h following the start of the experiment but routinely were given tap water and standard diet.

2.3. *Smooth Muscle Preparation*

The isolated intestinal smooth muscle preparation is one of the classical preparations in physiology and pharmacology for bioassays, or the study of drug action and autonomic control of motility. Since anesthetics can affect motility of the gut, rabbit was sacrificed by cervical dislocation, without use of anesthetic. The abdomen was cut open immediately and caecum was pulled forward to display the length of small intestine. The portion of intestine (jejunum, a section located near the end of the small intestine) was then removed and placed in a Petri dish or a beaker (Fig. 4) containing Tyrode's Ringer solution (Leod *et al.,* 1970). The rabbit was starved for 24 hours, so the gut segment was free of chyme. (However, if chyme is present, it can be removed by carefully flushing the segment with Tyrode's. A syringe filled with Tyrode's may be placed inside one end of the segment, and using slight pressure; the contents can be carefully forced out into a waste container. Caution must be exercised

because high pressure will severely damage the tissue.

2.4. *Isolated Intestine Segments Preparation*

The segments of small intestine (jejunum) about 3-4 cm long were dissected immediately from isolated intestine, later it was placed in petri dish containing Tyrode's ringer solution (Fig. 4). For experimentation, a piece of isolated muscle was mounted in a organ bath of 70 ml capacity, filled with Tyrode's Ringer solution (Fig. 3). Starting of the circulation was made with warm water through the organ baths at least 1/2 hour prior the experiment (Fig. 2). Organ bath circulating warm water temperature was maintained at 37°C throughout the experiment. The perfusion solution was bubbled with a mixture of 95% Oxygen and 5% CO_2 (Leod *et al.,* 1970). After noting the effect of the drug, draining, and refilling with fresh, warm Tyrode's solution was done. After the muscle contractions returned to the normal rhythmicity and tonus, next drug was introduced.

2.5. *In vitro Experiment*

The intestine segment was allowed to equilibrate before starting the experiment. After equilibration, drugs were administered in bath fluid to record their effect on Kymograph (Fig. 5). Every time responses of 2 successive doses of acetylcholine (ACh), at an interval of 10 min were recorded to confirm optimum responsiveness of the tissues. Spontaneous movements of intestine were recorded on oscillograph and Kymograph using isotonic transducer (Leod *et al.,* 1970). To determine the effects of plant extract on spontaneous movements of intestine, 0.1 g of dry crude aqueous extract of *CG* plant was dissolved in 1 ml of distilled water and thereafter, it was added to the organ bath after equilibrium period. The effect of aqueous leaves extract of medicinal plant *C. grandis* L. on contraction and relaxation pattern of isolated rabbit intestine (smooth muscle) was also observed (Fig. 1). Atropine and acetylcholine are used as standard drugs. Each tissue preparation was exposed to only one agonist or one combination. Atropine sulphate (1 µg/ml) was added to the bath fluid 15 min before recording the response of *CG* aqueous leaf extract (0.1 gm/ml) and ACh (2 ng/ml) to antagonise their effects.

Change in the spontaneous movement of jejunum was expressed as percent relaxation and percent contraction produced by the extracts and drugs used respectively according to the formula:

$$\text{Percentage change from control} = C+TNC\times T \times 100$$

Where,

C = Control readings

T = test readings after introducing drug

3. RESULTS AND DISCUSSION

The GIT is under control of the sympathetic and parasympathetic (autonomic nervous system). The relationship among these two arms keep balance and help to maintain the normal peristaltic functions responsible for the onward movements of intestinal contents (Osim, 2002). The parasympathetic nervous system uses chiefly acetylcholine (ACh) as its neurotransmitter.

The present study was carried out to evaluate the effect of aqueous extract of *Coccinia grandis* L. leaves on smooth muscles of rabbit jejunum *in vitro* experimental model and comparing it with the standard drug acetylcholine.

The smooth muscle contracted after administration of standard drug ACh. The muscle was then washed and after the contractions returned to its normal rhythmicity and tones,

Fig. 1. Water bath/Organ bath

Fig. 2. Segments of small intestine in petridish

Fig. 3. Isolated muscle mount in a muscle bath

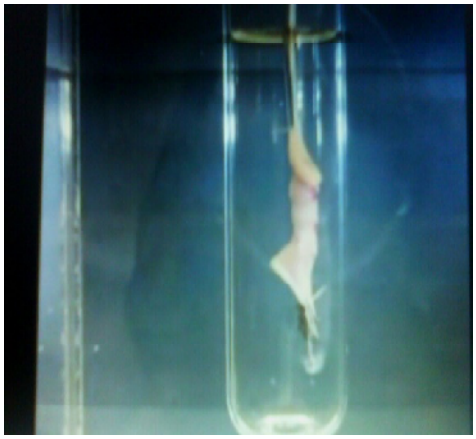

Fig. 4. Isolated piece of Jejunum (Contract and relax)

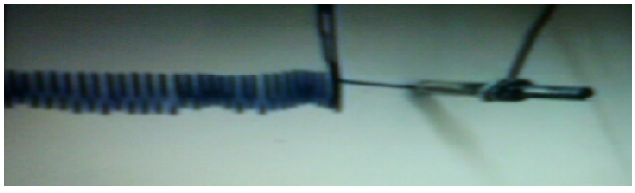

Fig. 5. Kymograph noting reading

aqueous extract was introduced. The responses of extract were dose dependent and fully reversible on wash. The findings suggested that the extract responses mediate through agonistic action without blockade of any receptor or enzyme. Extract possibly stimulates presynaptic cholinergic nerve endings to produce spasmodic response in rabbit jejunum smooth muscle. Atropine is a muscarinic receptor blocker. Hence, inhibition of contractile responses of ACh and *CG* aqueous leaf extract by atropine indicates that the responses of these two agonists were mediated through muscarinic receptors. This response also indicated that the plant contained an active ingredient that has spasmogenic response on rabbit jejunum smooth muscle.

Results showed that the *CG* aqueous extract produced an increase in intestinal transit as compared with the normal contractions of jejunum and with the standard drug i.e. acetylcholine. The number of contractions also increased after addition of extract. Result also indicated that the plant leaf extract possesses laxative activities along with spasmodic effect in the isolated tissues of rabbit, mediated partially through activation of muscarinic receptors; and the number of contractions were observed to be more pronounced that increased as compared to the standard drug. Thus, this study provides a rationale for the medicinal use of *C. grandis* L. leaves for the treatment of indigestion and constipation.

The results also showed that the aqueous extract of *CG* leaves has a significant spasmodic activity which supports its use in traditional herbal medicine practice. Results obtained indicate that *CG* aqueous leaves extract is safe and expected to be well tolerable after oral consumption.

Hence, the screening results of the aqueous leaf extract of the plant demonstrated that there is a remarkable increasing effect on the movement of the intestinal tissues of rabbit in comparison to controls (acetyl choline). The pattern of movement was observed as regularly irregular and an increase in contraction of smooth muscles.

The results of this *in vitro* study are summarized in Tables 1, 2 and 3.

4. CONCLUSION

The present *in vitro* study on *C. grandis* L. leaf aqueous extract supported that it can serve as potent anti-constipation drug having spasmodic effect on smooth muscles and hence

Table 1: Effect of Aqueous *C. grandis* L. Leaves Extract on Force of Contraction of Rabbit Jejunum

Plant Extract	No. of observation	Maximum contraction (cm) before extract administration	Maximum contraction (cm) after extract administration	Change observed	Percentage	Mean
Aqueous extract of *C. grandis* L. leaves	1	0.74	1.44	0.70	94.59%	92.64%
	2	0.86	1.64	0.78	90.69%	

Table 2: Effect of Aqueous *C. grandis* L. Leaves Extract on Force of Contraction of Rabbit Jejunum

Plant Extract	No. of observation	Rate of contraction before extract administration (No. of contraction/min.)	Rate of contraction after extract administration (No. of contraction/min.)	Conclusion showing effect on contraction
Aqueous extract of *C. grandis* L. leaves	1	13	14	Increases
	2	14	15	

Table 3: Effect of Aqueous *C. grandis* L. Leaves Extract on Force of Contraction of Rabbit Jejunum

Plant Extract	No. of observation	Pattern of contraction		
		Regular	Regularly irregular	Irregularly irregular
Aqueous extract of *C.grandis* L. leaves	1	—	Observed	—
	2	—	Observed	—

proven to serve as laxative drug against constipation conditions at large doses. This study is a substantial step and it further requires a long term study to evaluate its therapeutic efficacy and toxicity.

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Scientific Bases of Al-Hijamah or Cupping Therapy in Unani Medicine with Modern Techniques for the Treatment of Diseases Related to Uric Acid, Creatinine, Liver Enzymes and Lipid Profile

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Abstract

The present study, describes the effect of cupping therapy on variations observed and assessed in the biochemical parameters such as total cholesterol, triglycerides (TG), low density lipoprotein (LDL), glutamate pyruvate transaminase (GPT), alkaline phosphatase (ALP), total bilirubin, direct bilirubin, urea, creatinine and uric acid before and after cupping therapy.

Wet cupping therapy was undertaken in patients having 4 hrs fasting and after taking consent. The first session of wet cupping therapy was performed on both genders (n=69) aged between 20-65 years for 20-25 minutes on their first visit to the clinic followed by the second session after 1 week and all the biochemical parameters were recorded.

Different symptoms were assessed considering biochemical parameters.

Results of blood samples before and after 10 days of wet cupping treatment was statistically analyzed by applying paired t-test in this study. The values of serum total cholesterol and triglycerides were significantly lower except that of LDL. Thus, the results indicate that cupping therapy of 2 sessions is effective against high cholesterol and triglycerides levels but not for LDL indicating that probably more sessions are required.

Regarding tamperament the cupping therapy was most effective in patients having temperament sanguineous *harr* (hot) and *ratb* (moist) temperament and phlegmatic temperament i.e. *barid* (cold) and *ratb* (moist) whilst moderate improvement in bilious temperament *harr* (hot) and *yabis* (dry) and least in melancholic temperament that is *barid* (cold) and *yabis* (dry) was evident.

Keywords:

Wet Cupping, Blood Samples, Diagnostic Parameters, Evidenced Based Scientific Approach.

1. INTRODUCTION

According to WHO, Traditional Medicine (TM) is a comprehensive term used to refer to TM systems such as Traditional Chinese Medicine (TCM), Indian Ayurveda and Greco-Arab medicine and to various forms of

indigenous medicine. Traditional system of medicine include medication therapies if they involve use of herbal medicines, animal parts and/or minerals and non-medication therapies if they are carried out primarily without the use of medication, as in the case of acupuncture, manual and spiritual therapies (WHO, 2002).

According to Unani system of medicine management can be broadly classified into following therapies:

1. *Illaj Bil Ghiza* (Dietotherapy)
2. *Illaj Bil Dawa* (Pharmacotherapy)
3. *Illaj Bil Tadbeer* (Regimental Therapy)

1.1. *Illaj Bil Ghiza (Dietotherapy)*

Unani physician usually is a believer of particular diet plan for specific ailments. As for the preventive medicine in Unani system of medicine, it encompasses the famous *Asbab-e-Sitta Zaruria* (six essential factors) comprising of *Hawa-e-Muhit* (atmospheric air), *Makool-wa-Mashroob* (foods and drinks), *Harkat-wa-Sakoon-e-Jismani* (rest and physical activity), *Harkat-wa-Sakoon-e-Nafsani* (psychological activity and response), *Naum-wa-Yaqzah* (sleep and wakefulness), *Istifragh-wa-Ihtibas* (elimination and retention) for healthy lifestyle. One of the factor in it is *Makool wa Mashroob* (food and drink), Unani physicians use to recommend diet according to the temperament of the person, temperament of the disease, innate power, health and diseased state of the individual. Dietotherapy in Unani System operates in accordance with the rule of *Ilaj bil Zid* to relieve the ailment. Physicians recommend the diet of temperament opposing the existing ailment. *Ilaj bil Ghiza* (Dietotherapy) is recommended by alteration in the quantity and/or its quality depending on the nature of the disease (Look and Look, 1997).

1.2. *Illaj Bil Dawa (Pharmacotherapy)*

Unani System of Medicine emphasizes to maintain health through natural ways by changing lifestyle. In conditions where the other procedures are insufficient *Illaj Bil Dawa* (Pharmacotherapy) is adopted based on drug therapy in which active principle is not isolated instead whole plant is consumed counteracting the side effects.

Selection of drug therapy is determined by quality of drugs in relation to temperament, degree, quantity of drug indicating its weight/ efficacy and time of drug administration.

1.3. *Illaj Bil Tadbeer (Regimental Therapy)*

Ilaj bil Tadbeer (Regimental Therapy) is one of the most popular methods of treatment practiced by ancient Unani physicians since olden times comprising of special techniques or physical methods to improve the bodily constitution by removing waste materials hence stimulating the immune system of the body. As described by Unani physicians it can be classified into 9 groups: a) Cupping (*Hijamah*), b) Massage (*Dalak*), c) Exercise (*Riyazat*), d) Turkish bath (*Hammam*), e) Venesection (*Fasd*), f) Leeching (*Taleeque*), and g) Cauterization (*Aml-e-Kai*). In the Unani classical literature among all these regimental therapies, cupping is a widely discussed therapeutic regimen. According to the Unani System of Medicine, diseases occur due to the disproportionate distribution of humours or *Akhlat* (blood or dam, phlegm or balgham, bile or *safra,* black bile or *sauda*) inside the body. These humours, which are out of proportion (imbalanced), accumulate in various parts of the body producing inflammation and if persists may lead to various diseases (Ibn Sina, 1927).

As it has been proposed that cupping therapy enhances blood circulation, relieves

congestion and prevents the inflammatory extravasations (evasion of some body fluids e.g. blood) from the tissues (Chirali, 1999 and Yoo *et al.,* 2004, Qureshi and Hannan, 2016). In case of wet cupping therapy its outcome depends on various parameters such as: pressure applied, duration, number of cups used, frequency of cupping therapy and size of capillary fenestrae and pores. Skin capillary pores diameters (12 nm) and fenestrae (60-80 nm) are similar to that of glomerular capillary pores diameter (15 nm) and fenestrae (65 nm). Thus only particles in nanometer less than the range can be filtered through skin capillaries (percutaneous route) and excreted including damaged cells or their debris. However, normal red blood cells (RBCs), white blood cells (WBCs) and platelets in micrometer range cannot leak through capillary pores (Sarin, 2010; Them *et al.,* 2002).

Human skin is composed of epidermis and dermis. The epidermis is the outermost, cellular and non-vascular layer of the skin with thickness ranging from 0.07 mm to 0.12 mm (0.8 to 1.4 mm in the palms and soles). The epidermis is impermeable to water and has protecting barrier functions against physical, ultraviolet injury, chemicals and microbial penetration. The second layer of the skin dermis is the vascular connective tissue matrix that interacts with the epidermis. Dermis is strong, elastic, thin (1 mm to 3 mm) and can store water. The depth of human skin is relatively thin (average 2 mm) and is richly supplied by a network of capillaries. During cupping therapy it can tolerate mechanical stress through migration of the cells originating at the basement membrane to the above layers. Thus wet cupping therapy facilitates blood clearance through filtration of circulating blood via rich capillary network. (Fawcett 1986; Sanders *et al.,* 1995).

2. MATERIAL AND METHODS

2.1. *Study Design*

The present study was conducted during November 2012 to December 2014 at Shifa-ul-Mulk Memorial Hospital, Madinat-al Hikmah, and Eastern Specialist Clinic (PECHS), Karachi, Pakistan in collaboration with Brookes Health and Education Foundation.

It has been described previously in detail (Qureshi and Hannan, 2016). Additionally, in this study the blood samples from the subjects were collected for biochemical analysis and other parameters such as age, fasting state and disease conditions were also considered.

2.2. *Requirements and Equipments*

Self-designed cupping chair, bell shaped small (30 ml) or larger (60 ml) vacuum cupping cups of circumference from 25 mm-75 mm, vacuum pump with pistol grip (U.S. Global, Karachi), sterilized and disposal items included: gloves, surgical blade, cotton and medical gauze and micro-pore surgical tape (Medics shop at medicine market, Karachi), pyodine (Brookes Pharma, Pakistan) and honey (Al-shifa) were purchased from the local market.

2.3. *Inclusion Criteria*

The patients (n=69) having disturbed cholesterol (n=8), triglycerides (n=8), LDL (n=7), total bilirubin (n=5), direct bilirubin (n=6), SGPT (n=10), ALP(n=3), uric acid (n=8), urea (n=9) and creatinine (n=5) both genders aged between 20-65 years were enrolled in the trial after obtaining their informed consent.

Temperamental Evaluation:

The temperament of their patients was assessed as described early with slight modifications.

2.4. *Exclusion Criteria*

Patients suffering from chronic serious

illnesses, dehydration, diarrhea, hypertension, severe vomiting and uncontrolled diabetes, females during menstruation and pregnant women were excluded. Cupping was also avoided in patients experiencing bleeding disorders, inflammation, skin infection, ulcer, or prone to bruising.

2.5. *Instructions for the Patients*

Prior to the cupping treatment regimen all the patients were briefed as described earlier:

i) Briefly, patients were asked to fast and have a bath 4 hrs before cupping therapy.
ii) Patient undergo full investigation related to the disease especially diabetes. The requirement of biochemical assays and pathological data was completed before the initiation of therapy.
iii) Physical examination included recording of body temperature, pulse rate, respiratory rate and blood pressure.
iv) Abstain from consuming dairy products, food intake and sleeping at least 1-2 hrs on the day of treatment after therapy.
v) Strenuous exercise, coitus and blood thinning medicines are forbidden prior to cupping therapy.

2.6. *Physical Examination*

Patients were examined to declare capable of bearing the procedure. Site of pain was investigated for inflammation, swelling, sensitivity and vascularity of the area.

2.7. *Blood Specimen Collection and Processing*

From each subject venous blood samples were collected in sterile 1 ml test tube before and after 10 days of wet cupping therapy through venipuncture, by placing the tourniquet 3 to 4 inches above the selected puncture site on the patient, which should not be too tight or not to leave it longer than 1 minute. The sample was centrifuged for 450 rmp/minute, the recommended spin time is 10 minutes and the serum was used for biochemical analyses.

2.8. *Biochemical Parameters Assessment in Serum*

The total cholesterol, triglycerides (TG), low density lipoprotein (LDL), glutamate pyruvate transaminase (GPT), alkaline phosphatase (ALP), total bilirubin, direct bilirubin, urea, creatinine and uric acid were determined using Merck Kits Germany.

In case of LFT's R1 (1 ml/1000 µ) and R2 (1 ml/250 µ) reagents were used making 1:4 dilutions. For bilirubin R1 (50 µl), R2 (200 µl), R3 (1 ml) and R4 (1 ml) reagents were employed, whereas, for lipid profile, urea and creatinine 1 ml reagent is added in 10 µ of serum and incubate for 10 minutes afterwards the results are analyzed using Merck Micro Lab-300.

3. RESULTS AND DISCUSSION

Nowadays, wet-cupping therapy is popularly used worldwide and apparently facilitates the regulation of various systems in the body including neurotransmitters and hormones. In the hematological system, its main effect is regulation of coagulation and anti-coagulation systems, reduction of hematocrit (HCT) thereby increasing the flow of oxygenated blood to various organs. It also effects the immune system inducing local inflammation followed by activation of the complementary system with increase in the levels of interferon and tumor necrosis factor (TNF), affects the thymus thus, regulating and increasing the flow of lymph in lymphatic system (Rozegari, 2000, Ahmadi *et al.,* 2008).

Despite the popularity of wet cupping therapy its mechanism of action is not clear.

However, it has been proposed to act through oxidative balance as the venous blood has higher activity of myeloperoxidase (MPO) and of nitric oxide (NOx) while lower activity of superoxide dismutase (SOD) (Fairoz, 2010). It modulates the immune cellular conditions particularly of innate immune response NK cell % and adaptive cellular immune response SIL-2R (Ahmed, *et al.,* 2005).

The reason that cupping works may be due to its physiological affect by either stimulating or relaxing the body by evacuation through bleeding (Gutteridge, 1992).

The clinical studies published between 1959 and 2008 on cupping therapy including 73 randomized controlled trials (RCTs), 22 clinical controlled trials, 373 case series and 82 case reports supported the progress in various diseases particularly pain conditions and herpes zoster etc. No serious adverse effects were reported in these studies (Cao *et al.,* 2010). According to these results, quality and quantity of RCTs on cupping therapy appeared to be improved during the past 50 years. However, further rigorous designed trials in relevant conditions are required to support their use in practice.

Considering important diagnostic biochemical parameters including blood lipid profile in which cholesterol and triglycerides determines the risk of plaques formation in the arteries that can lead to atherosclerosis by narrowing or blockade of arteries whereas, high cholesterol levels often is considered to be a risk factor for heart disease. Wet cupping therapy has been reported to relieves hyperlipedmic conditions and hence prevent atherosclerosis.

In the present study result of blood samples before and after 10 days of wet cupping treatment was statistically analyzed by applying paired t-test. The values of serum total cholesterol ($p=0.016$) and triglycerides ($p=0.028$) were significantly lower except that of LDL ($p=0.069$). Thus, the results indicate that cupping therapy of 2 sessions is effective against high cholesterol and triglycerides levels but not for LDL levels indicating more sessions of cupping therapy are required for prolonged duration to achieve the normal ranges in order to relieve disease symptoms.

In Table 1 the lipid profile including serum total cholesterol, triglycerides (TG), low density lipoprotein (LDL) before and after cupping is presented. The magnitude of decline in the levels of above parameters were compared with the standard ranges and represented as normal (N), borderline (B) and high (H). A general decline in cholesterol levels in patients was observed with H, B and N levels i.e. in 2 cases high cholesterol levels were reduced by 35 and 24 points from 243 mg/dl to 208 mg/dl and 289 mg/dl to 265 mg/dl respectively however, it remained in high cholesterol level category. 3 cases of normal cholesterol decline from 128 to 115, 139 to 130 and 173 to 106 with the decline of 13, 9 and 67 points which was highest decline (67 points) in individuals with normal levels of cholesterol.1 case of borderline cholesterol 194 decline to still borderline 190. 1 case of borderline cholesterol 200 decline to 185.

For TG levels a dramatic decline of 102 points was noted in patients with high (H) points but it still remained higher. 2 cases of high (H) TGs was declined from 303 to 201 and 255 to 239 by 102 and 16 points. 5 cases of normal TGs declined from 122 to 103, 120 to 101, 127 to 122, 113 to 80 LDL showed reduction by 70 points with a shift from higher (HR) to borderline (B) after cupping therapy.

In some cases the level before cupping therapy whether it is high (H), borderline (B) or normal (N) declined with improvement in the overall symptoms (fatigue, in digestion and

numbness) indicating improvement towards better and healthy lifestyle conditions.

In previous research studies, relationship between some blood parameters and wet cupping therapy, showed that cupping can only regulate some blood parameters such as Cholesterol, HDL, LDL, and FBS in young healthy male (20-27 years old) after five sessions of cupping (one time per month) (Ranaei-siadat *et al.,* 2004).

In another study results showed that subjects treated with one session of cupping therapy declared significant increase ($P< 0.0000$) in LDL cholesterol concluding that cupping will increase the level of LDL cholesterol an hour after treatment (Gugun & Alfian, 2010).

Another biochemical parameter evaluated for determining efficacy of cupping therapy was Liver Function Tests (LFTs). LFTs are not specific to specific systems or disease processes, yet abnormalities may indicate significant or serious diseases. Abnormal LFTs are used to diagnose any underlying liver disease, however, single abnormalities in LFTs are difficult to localize and diagnose. Bilirubin blood levels may become elevated with impaired bile flow. This can occur in severe liver disease, gallbladder disease, or other bile system conditions. Usually either alanine aminotransferase (ALT) or aspartate aminotransferase (AST) is measured. These proteins both indicate leakage from damaged cells due to inflammation or cell death. Liver disease is more likely to occur when the values of AST and ALT are higher, ALT rises more than AST in acute liver damage. Raised GGT in patients with chronic liver disease is associated with bile duct damage and fibrosis. If the GGT concentration is normal, a high ALP result suggests bone disease. ALP is physiologically increased when there is increased bone development (e.g. adolescence) and is elevated in the third trimester of pregnancy (produced by the placenta) (Giannini *et al.,* 2005; Rochling 2001; Limdi *et al.,* 2003; Sherwood *et al.,* 2001 and Walsh *et al.,* 2000).

The most sensitive and widely used LFTs liver enzymes are the aminotransferases including aspartate aminotransferase (AST or SGOT) and alanine aminotransferase (ALT or SGPT). AST (SGOT) is normally found in multiple tissues including liver, heart, muscle, kidney, and brain. It is released into serum when any one of these tissues is damaged (Niasari, *et al.,* 2007). One of the research study showed that blood sampling 2 weeks after cupping therapy did not cause any change in SGOT level but there was significant increase of SGPT after 2 weeks. The effect of cupping therapy improving blood/lymph circulation and strengthening organic (liver) function was introduced in the study of Jun-Ru (Jun-ru *et al.,* 2007). Such finding is not in accordance that liver damage causes SGPT increase (Wong *et al.,* 2000) i.e. it seems the augmentation of SGPT after cupping therapy is not as a result of liver impairment but more probably other factors like skin injury after cupping are related to this increase.

Table 2 indicates that the bilirubin, serum glutamyl phosphate transaminase (SGPT) and alkaline phosphatase level declined considerably as seen in case of total bilirubin 4 cases of borderline (B) were decrease to 0.1 points that is from 0.9 to 0.8 (3 cases), 1.0 to 0.9 (1 case) but still remained at borderline levels. In case of direct bilirubin 1 case of high (H) level declined by 0.01 points from 0.16 to 0.15 levels but still remained high. 1 case of high (H) level was declined to normal (N) from 0.15(H) to 0.06 (N) by 0.09 points. 4 cases of normal still declined by 0.02 points from 0.06 to 0.04, 0.06 to 0.05, 0.06 to 0.04, 0.04 to 0.02.

TABLE 1: Effect of Cupping Therapy on Lipid Profile in Patients

S.No.	Cholesterol			Triglycerides (TG)			Lower Density Lipoprotein (LDL)			Comments by the patients
	Before	After	Comments after treatment	Before	After	Comments after treatment	Before	After	Comments after treatment	
1.	243 (H)	208 (H)	Level declined by 35 points	303 (H)	201 (H)	Level declined by 102 points	179 (H)	109 (B)	Level declined by 70 points	Improvement in symptoms of fatigue and digestion.
2.	202 (H)	188 (N)	Level declined by 14 points	255 (H)	239 (H)	Level declined by 16 points	142 (H)	140 (H)	Level declined by 2 points	Improvement in symptoms of fatigue, numbness and digestion
3.	194 (B)	190 (B)	Level declined by 4 points	122 (N)	103 (N)	Level declined by 19 points	129 (H)	106 (B)	Level declined by 23 points	Improvement in symptoms of fatigue and digestion.
4.	128 (N)	115 (N)	Level declined by 13 points	120 (N)	101 (N)	Level declined by 19 points	130 (H)	119 (B)	Level declined by 11 points	Improvement in symptoms of fatigue and digestion.
5.	139 (N)	130 (N)	Level declined by 9 points	127 (N)	122 (N)	Level declined by 5 points	51 (N)	45 (N)	Level declined by 6 points	Improvement in symptoms of fatigue and digestion
6.	289 (H)	265 (H)	Level declined by 24 points	113 (N)	80 (N)	Level declined by 33 points	66 (N)	57 (N)	Level declined by 9 points	Improvement in symptoms of fatigue, exhaustion and digestion
7.	173 (N)	106 (N)	Level declined by 67 points	63 (N)	57 (N)	Level declined by 6 points	220 (H)	205 (H)	Level declined by 15 points	Improvement in symptoms of fatigue and digestion
8.	200 (B)	185 (N)	Level declined by 15 points	166 (B)	108 (N)	Level declined by 58 points				Improvement in symptoms of fatigue, exhaustion and digestion
Mean± SEM	196	173		158	126		131	111		
n	**8**			**8**			**7**			
P-value	.016			.028			.069			

Diagnostic range for triglycerides (TG), cholesterol, and low density lipoprotein (LDL).
Cholesterol: Normal (N) = below 200 mg/dl, Borderline (B) = 200-239 mg/dl and High (H) = 240 and above mg/dl.
Triglycerides: Optimal = Less than 100 mg/dl, Normal = 101-150 mg/dl, Borderline = 150-199 mg/dl and High = 200-499 mg/dl.
Lower Density Lipoprotein (LDL): Normal (N) = Below 100 mg/dl, Borderline (B) = 130-159 mg/dl and High (H) = 160-189 mg/dl.
(Friedewald *et al.,* 1972; Tanno *at al.,* 2010 and Mora *et al.,* 2009).

Table 2: Representing the Variations in LFT's Before and After Cupping Therapy

	(TBL)*			(DBL)**			(SGPT)***			(ALP)****		
S.No.	**Before**	**After**	**Comments**	**Before**	**After**	**Comments**	**Before**	**After**	**Comments**	**Before**	**After**	**Comments**
1.	0.9 (B)	0.8 (B)	Level declined by 0.1 points	0.16 (H)	0.15 (H)	Level declined by 0.01 points	58 (H)	46 (N)	Level declined by 12 points	182 (HR)	143 (H)	Level declined by 39 points
2.	1.0 (B)	0.9 (B)	Level declined by 0.1 points	0.06 (N)	0.04(N)	Level declined by 0.02 points	31 (N)	30 (N)	Level declined by 1 point	198 (HR)	129 (H)	Level declined by 69 points
3.	0.9 (B)	0.8 (B)	Level declined by 0.1 points	0.15 (H)	0.06 (N)	Level declined by 0.09 points	32 (N)	25 (N)	Level declined by 7 points	240 (HR)	190 (HR)	Level declined by 50 points
4.	0.8 (B)	0.7 (N)	Level declined by 0.1 points	0.06 (N)	0.05 (N)	Level declined by 0.01 points	42 (N)	37 (N)	Level declined by 5 points			
5.	0.98 (B)	0.82 (B)	Level declined by 0.16 points	0.06 (N)	0.04(N)	Level declined by 0.02 points	15 (N)	12 (N)	Level declined by 3 points			
6.				0.04(N)	0.02 (N)	Level declined by 0.02 points	55 (B)	51 (N)	Level declined by 4 points			
7.							70 (HR)	60 (H)	Level declined by 10 points			
8.							27 (N)	21 (N)	Level declined by 6 points			
9							90 (HR)	55 (B)	Level declined by 35 points			
10							72 (HR)	44(N)	Level declined by 22 points			
Mean± SEM	0.916	0.804		0.08	0.06		49.2	38.1		206	154	
n	**5**			**6**			**10**			**3**		
P-value	.001			.073			.013			.027		

Normal blood test results for typical liver function tests include:

ALP: 45 to 115 U/L, Total Bilirubin:. 0.3 to 1.0 mg/dl, Direct bilirubin: 0 to 0.4 mg, Glutamic-pyruvic transaminase SGPT: Normal = 7 to 55 units per liter (U/L), Borderline 2-3 times higher than normal range and High = 1000 and above

Alkaline phosphatase ALP. Normal 45 to 115 U/L.

ALT (or SGPT serum glutamic-pyruvic transaminase) is found particularly in large amounts in the liver and plays an important role in metabolism that converts food into energy. The ALT test may be recommended if child is experiencing symptoms of liver disease, including jaundice (yellowish skin or eyes), dark urine, nausea, vomiting, or abdominal pain. It also be recommended to diagnose infections of the liver such as viral hepatitis (ALT levels are high with acute hepatitis) or to monitor patients using medications that cause liver-related side effects, or to evaluate an injury to the liver.

The damaged liver cells release excessive ALP in the blood. This test is often used to detect blocked bile ducts because ALP is especially high in the edges of cells that join to form bile ducts.

Usually, a chemical test is used to first measure the total bilirubin level (unconjugated plus conjugated bilirubin). If the total bilirubin level is increased, a second chemical test is used to detect water-soluble forms of bilirubin, called direct bilirubin. The direct bilirubin test provides an estimate of the amount of conjugated bilirubin present. Subtracting direct bilirubin level from the total bilirubin level estimate the indirect level of unconjugated bilirubin. In adults and older children, bilirubin is measured to:

a)Diagnose and/or monitor diseases of the liver and bile duct (e.g., cirrhosis, hepatitis, or gallstones).

b)Evaluate sickle cell disease or other causes of hemolytic anemia; in case of excessive RBC destruction.

*Total Bilirubin, **Direct Bilirubin, ***Serum Glutamyl Phosphate Tansaminase, ****Alkaline Phosphatase

TABLE 3: Representing the Variations in Renal Function Before and After Cupping Therapy

S.No.	Uric Acid (UA)			Urea (UR)			Creatinine (CR)		
	Before	After	Comments	Before	After	Comments	Before	After	Comments
1.	4.9 (N)	4.2 (N)	Level declined by 0.7 points	26 (N)	22 (N)	Level declined by 4 points	0.6 (N)	0.5 (N)	Level declined by 0.1 points
2.	6.0 (N)	5.0 (N)	Level declined by 0.1 points	30 (N)	27 (N)	Level declined by 3 points	0.9 (N)	0.8 (N)	Level declined by 0.1 points
3.	7.1 (N)	6.2 (N)	Level declined by 0.9 points	21 (N)	18 (N)	Level declined by 3 points	1.1 (B)	0.7 (N)	Level declined by 0.4 points
4.	4.8 (N)	4.7 (N)	Level declined by 0.1 points	24 (N)	20 (N)	Level declined by 4 points	1.5 (H)	1.3 (N)	Level declined by 0.2 points
5.	6.5 (N)	6.3 (N)	Level declined by 0.2 points	44 (H)	35 (N)	Level declined by 9 points	0.8 (N)	0.6 (N)	Level declined by 0.2 points
6.	4.3 (N)	4.1 (N)	Level declined by 0.2 points	37 (N)	30 (N)	Level declined by 7 points			
7.	4.2 (N)	3.9 (N)	Level declined by 0.3 points	30 (N)	21 (N)	Level declined by 9 points			
8.	3.8 (N)	3.7 (N)	Level declined by 0.1 points	24 (N)	18 (N)	Level declined by 6 points			
9.				38 (B)	25 (N)	Level declined by 13 points			
Mean± SEM	5.2	4.7		30.4	24		0.98	0.78	
n	**8**			**9**			**5**		
P-value	.012			.000			.022		

Creatinine-Low levels are sometimes seen in kidney damage, protein starvation, liver disease or pregnancy. Elevated levels are sometimes seen in kidney diseases involved in impairment of kidney function as excreting excessive creatinine, muscle degeneration and some drugs.

Creatinine, serum = 0.6-1.2 mg/dl, Creatinine, Urine (male) = 0.8-2.4 g/24 hrs, Creatinine, Urine (female) = 0.6-1.8 g/24 hrs, Creatinine clearance (male) = 97-137 mL/min, Creatinine clearance (female) = 88-128 mL/min.

Uric acid-High levels are noted in gout, kidney disease, alcoholism, high protein diets and toxemia in pregnancy. Low levels may be indicative of kidney diseases as malabsorption, poor diet, liver damage or an acidic kidney.

Uric Acid, serum = 3.0-8.2 mg/dL, urine = varies with diet

Urea is an end product of protein metabolism and its normal range in blood is 20-40 mg/dl.

In SGPT 1 case of high (H) declined to normal (N) from 58 to 46 by 12 points. 1 case of borderline (B) declined to normal (N) from 55 to 51 by 4 points. 1 case of higher level (HR) was declined to high (H) from 70 to 60 by 10 points. 1 case of higher level (HR) was declined to borderline (B) from 90 to 55 by 35 points. 1 case of higher (HR) level was declined to normal (N) from 72 to 44 by 22 points. 5 cases of normal (N) SGPT were further declined from 31 to 30, 32 to 25, 42 to 37, 15 to 12 and 27 to 21 by 1-7 points.

Whereas, for 2 cases of alkaline phosphatase higher (HR) level were declined to high (H) level from 182 to 143 and 198 to 129 by 39 and 69 points. 1 case of alkaline phosphatase with higher (HR) level remained still at higher (HR) level but delined from 240 to 190 by 50 points.

For the parameters total bilirubin, direct bilirubin, SGPT and alkaline phosphatase before and after cupping therapy were also analyzed by applying statistical tool paired t test and found that the *p*-value showed significant difference except for direct bilirubin (0.073). All showed highly significant *p*-value in order of: total bilirubin (0.001), SGPT (0.013) and ALP (0.027).

One of the biochemical indicators that were used to assess efficacy of cupping therapy was uric acid. Uric acid is an end product of the metabolism of purine produced by the action of xanthine dehydrogenase or xanthine oxidase. It is present in blood and excreted in the urine. It has been found that the high level of serum uric acid is associated with various illnesses including hypertension, atherosclerosis, vascular anomalies, hyperinsulinemia and renal insufficiency. Uric acid is a factor involved in different pathogenic courses and may have potential value for the assessment of variations in clinical settings and prognosis of illness. Serum creatinine is the most commonly used diagnostic indicator of renal function. Urea also reflects the renal function and increases when renal function declines. Studies from the general population suggest that obesity may also produce renal insufficiency in individuals without hypertension, diabetes, or preexisting renal disease (Nakagawa *et al.,* 2008).

In case of uric acid, 8 cases of normal (N) uric acid was still declined from 4.9 to 4.2, 6.0 to 5.0, 7.1 to 6.2, 4.8 to 4.7, 6.5 to 6.3, 4.3 to 4.1, 4.2 to 3.9 and 3.8 to 3.7 by 0.1 to 0.7 points. 7 cases of normal (N) urea still declined from 26 to 22, 30 to 27, 21 to 18, 24 to 20, 37 to 30, 30 to 21 and 24 to 18 by 3 to 9 points. 1 case of high (H) urea was declined to normal (N) from 44 to 35 by 9 points. 1 case of borderline (B) urea was declined to normal (N) from 38 to 25 by 13 points. 3 cases of normal (N) creatinine was still declined from 0.6 to 0.5, 0.9 to 0.8, and 0.8 to 0.6 by 0.1 to 0.2 points. 1 case of borderline (B) creatinine was declined to normal (N) level from 1.1 to 0.7 by 0.4 points. 1 case of high (H) level was declined to normal (N) level from 1.5 to 1.3 by 0.2 points. In this study reduced blood levels of urea (0.000), uric acid (0.012) and creatinine (0.022) showing highly significant difference regarding urea (Table 3).

It can be concluded that biochemical parameters (lipid profile, renal parameters, liver enzymes) as shown in Tables 1, 2 and 3 showed significant decrease in cholesterol, triglycerides, total bilirubin, SGPT, alkaline phosphatase, uric acid, urea, creatinine levels before and after cupping therapy except LDL and direct bilirubin levels.

It is noteworthy that marked improvement was linked with the temperament of patients such as sanguineous, phlegmatic, bilious, melancholic and the disease conditions were improved as > sanguineous temperament *harr* (hot) and *ratb* (moist) > phlegmatic

temperament *barid* (cold) and *ratb* (moist) > bilious temperament *harr* (hot) and *yabis* (dry) > melancholic temperament , *barid* (cold) and *yabis* (dry). The therapeutic effect of wet cupping therapy followed a sequence of improvement in biochemical parameters as: Cholesterol > triglycerides > alkaline phosphatase > SGPT > bilirubin > urea > uric acid > creatinine.

It has been noticed that temperamental imbalance of cold and dry qualities, results in increased stiffness aggravating cold and dry qualities of the connective tissues. Other associated factors like increased weight, hormonal variations, excessive intake of cold and dry foods can also take part in aggravating the disease. Additionally, six essential factors of Unani medicine play an important role in progression towards temperamental imbalance. In the present study, patients having sanguineous and phlegmatic temperament a marked improvement was observed in their ill-health conditions, improved sleeping pattern as well as their emotional status.

One of the studies showed that there might be some imbalance or impurities present in the blood which is evacuated or discarded through cupping therapy resulting in a favorable balance environment including various vital parameters (Bilal Alam Khan *et al.,* 2011).

The above mentioned facts obtained as a result of wet cupping therapy indicated that it is very effective and promising technique against cardiovascular disease, liver diseases, obesity and also improved kidney function as reflected by the reduction in the serum cholesterol, creatinine, triglyceride, urea, uric acid levels and liver function tests. The patients having sanguineous *harr* (hot) and *ratb* (moist) and phlegmatic temperament *barid* (cold) and *ratb* (moist) showed marked improvement whilst moderate improvement in bilious temperament *harr* (hot) and *yabis* (dry) and least in melancholic temperament that is *barid* (cold) and *yabis* (dry) is seen. These results led us to conclude that it is a simple, safe and effective way of treatment to be used against afore-mentioned diseases. However, it is a preliminary report on comparatively small scale and needs to be extended in future to a larger sample size before definite conclusion could be reached.

However, patients showing no response to cupping therapy were either affected by the prolonged medication or severity of the disease indicating marked disability or any other organic disorder.

Conflict of Interest

The authors declare that there is no conflict of interest.

Authors Contribution

Tasneem Qureshi: Conducted the therapy.

Hakim Zahoor: Conducted the therapy.

Prof. Dr. Hakim Abdul Hannan: supervised the research study.

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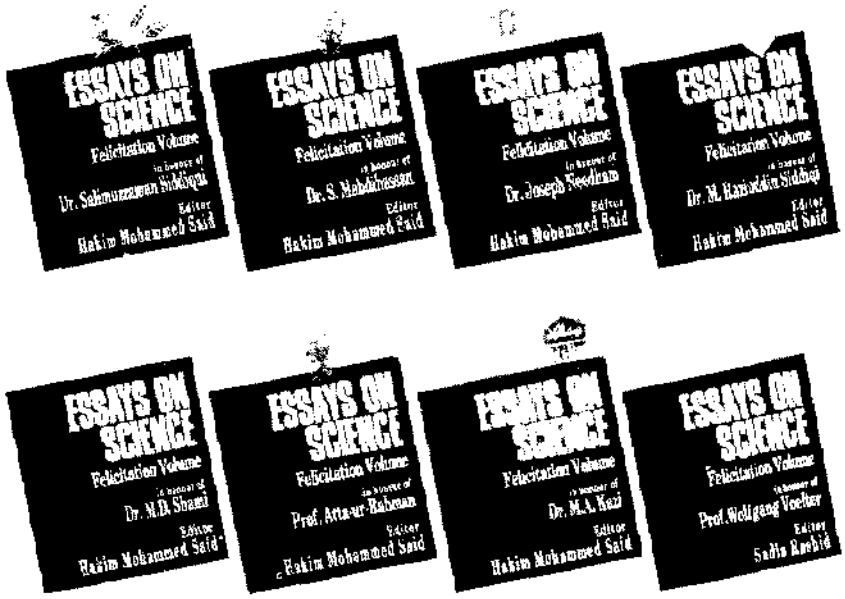

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