Hamdard Medicus

January-March 2017
Vol. 60, No. 1
Patron-in-Chief: Mrs. Sadia Rashid

Quarterly Journal of Science and Medicine

Published by

Hamdard University

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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.

HAMDARD MEDICUS

Quarterly Journal of Science and Medicine

Regd. No. M-73. International Standard Serial Number (ISSN) 0250-7188

Vol. 60 **January-March 2017** **No. 1**

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CONTENTS

HAMDARD MEDICUS

Quarterly Journal of Science and Medicine

Vol. 60 **January-March 2017** **No. 1**

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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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(Books) 1. Gaill, W., Jon, A.W. (1995). *Manual of Clinical Microbiology*. ASM Press, Washington, DC. 6th Edn., pp. 1327-1332.
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Unani Treatment for the Removal of Renal and Vesicle Calculi (*Hisat-e-Kulia wa Masana*) – A Case Report

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Abstract

Hisat-e-Kulia wa Masana (Renal and vesicle calculus) is a hard mass, formed from the crystals that aggregated on the inner surfaces of the kidney. This is a case report of a 57 years old male OPD patient of Shifa-ul-Mulk Memorial Hospital for Eastern Medicine, Hamdard University with lumbar pain. The patient had a history of recurrent lower urinary tract infection accompanied by frequent urination and its urgency, difficulty in voiding, small amount of urinary stream. Patient was a known case of diabetes and ischemic heart disease. Physical examination revealed mild tenderness in left lumbar region. According to laboratory examination profile, blood urea (38 mg/dl) and creatinine (0.8 mg/dl) were within normal limits; however, hemogram demonstrated reactive neutrophilic leukocytosis. Renal ultrasound showed single calculus measuring 0.4 cm in left kidney while urinary bladder showed multiple calculi (largest ranging from 0.7 and 0.8 cm) along with enlarged prostate weight (38.3 g). The patient received polyherbal decoction of Unani codded formulation NU-08 and *Hab-e-Rasoot* (1 tablet twice a day) for 21 days. This treatment was successful and resulted in excretion of one tiny and one large stone (~1 cm) through urination. This was further confirmed by ultrasound.

Keywords

Hisat-e-Kulia wa Masana, Renal and vesicle calculus, Unani treatment, Case report.

1. INTRODUCTION

According to Unani philosophy, renal calculi are formed due to the excessive amount of abnormal heat (hotness) resulting from imbalanced internal or external environment. It refers to accumulation of viscous and turbid matter comprising possibly of phelgum, pus or blood. The excessive amount of heat absorbs the moistness, producing abnormal dryness. This entire process of increased dryness leads to incinerate/decomposed substance(s) or reduced to ashes resulting in calculi formation. If the passage or junction throughout the renal system is constricted due to viscous matter, obstruction of the spaces may occur that hinders urination which may be excreted in dribbling manner, resulting in oliguria. In case of concretion the causative matter is less viscous so it can easily pass through urinary system (Ahmed, 1969).

Kidney stones are small, hard deposits of calcium or a combination of calcium and oxalate that can be formed in renal pelvis. These may enter into ureter and referred to as ureteral stones. Many of these stones are less than 5 mm in size which travel to the bladder in just a few days or weeks without any treatment,

and exit the body through urine. In certain conditions where stones are smaller use of painkillers, intake of sufficient water and other fluids facilitate passage of stones through kidneys. However, larger stones above 5 mm may be trapped while exiting renal pelvis or require longer duration to move through the ureter, thereby causing severe pain and accompanied by other symptoms such as urine retention, dysuria and hematuria (Miller and Lingeman, 2007). Renal stone may get stuck at various sites such as: 1) Renal pelvi-uretric junction, 2) Where iliac artery cross over the ureter or 3) Where the vasdefrens in males and broad ligament in females cross over the ureter, 4) Where the ureter enters into the bladder, 5) Posterior/distal uretric orifice and 6) in the bladder, the stone may get trapped in the bladder neck area. These stones either passed spontaneously or required to be broken by sound waves or removed surgically. The most suitable treatment depends on the size, the type and the position of the stones in the kidney or the urinary tract system.

The main symptom of kidney stones is pain emerging as uncomfortable feeling leading to waves of severe pain. The pain may affect lower abdomen, belly or back "flank pain," depending on the position of stone in the ureter. The pain is especially severe when they travel through a narrower passage of the ureter, for example during its journey from the renal pelvis into either ureter or bladder. Sudden episodes of severe pain on one side radiating through lower abdomen are common and referred to as renal colic. Its intensity gets stronger and weaker in waves, sometimes accompanied by nausea and vomiting. Some people also toss and turn to find a position that helps to relieve the pain. An episode of renal colic usually lasts from 20 to 60 minutes. Other possible symptoms of a kidney stone passing through the ureter includes painful urination, blood in urine, and a stronger or more frequent urge to urinate. Sometimes the pain may extend to genitals.

1.1. *Composition of Kidney Stones*

Kidney stones are made up of the dissolved salts present in urine. Once it is supersaturated, the formation of crystals is initiated that over the period of time develops into stones. Most common kidney stones are as follows:

1) Calcium stones: About 80% of kidney stones are made up of calcium salts – calcium oxalate or calcium phosphate.

2) Uric acid stones: Uric acid is the cause of about 5 to 10% of all kidney stones.

3) Struvite stones: About 10% of kidney stones are made up of the mineral struvite, which contains mostly magnesium and phosphate (Malvinder, 2004).

1.2. *Prevalence*

The epidemiology of renal and vesicle calculus varies geographically due to socioeconomic, environmental, lifestyle, and climatic conditions. Pakistan is situated in the geographical region known as "stone belt" stretching from Egypt and Sudan through the Middle East, India, Pakistan, Burma, Thailand, Indonesia and Philippines reporting consistently high incidence of urolithiasis. (Robertson 1984). In Pakistan due to lack of centralized epidemiological data, it is estimated that ~40-50% of the patients in urological wards of major hospitals suffer from kidney stones (Hussain *et al.,* 1998). In economically developed countries the prevalence rate ranged between 4% and 20%. In Germany, an increase in the prevalence and incidence of urolithiasis from 4% to 4.7% has been reported. On the contrary, according to National Health and Nutrition Examination Survey II and III, renal stone

prevalence among 20 to 74 old US residents in 1976 to 1980 (3.8%) was increased in 1988 to 1994 (5.2%), and it was greater in males than females (Stamatelou *et al.,* 2003). In developing countries the prevalence of stones is probably underestimated as silent and not yet discovered kidney stones were diagnosed by renal sonography in 3% of non-symptomatic subjects (Buchholz *et al.,* 2003).

Renal stones are usually more frequent in men. It is well accepted that greater intake of dietary calcium, potassium, and total fluid reduce the risk of kidney stone formation, while supplemental calcium, sodium, animal protein, and sucrose may increase the risk. However, some dietary risk factors may differ with age and sex. As in younger women a higher consumption of dietary calcium reduces the risk of kidney stone formation, while supplemental calcium increases the risk of stone formation. Phytate along with less intake of water in either cases and fluid are also associated with a reduced risk of stone formation whereas, other dietary factors, such as animal protein and sucrose increases the risk of stone incidence. Also in older adults the relation between diet and kidney stones may be different because the metabolism of many dietary factors, such as calcium, may change with age (Curhan *et al.,* 2004). Some diseases such as Type 2 diabetes, obesity, and hypertension are associated with nephrolithiasis, whereas diabetic condition may be considered as an additional factor in the development of uric acid stones (Lieske *et al.,* 2006). Insulin resistance, characteristic of the metabolic syndrome and Type 2 diabetes, leads to low urine pH through impaired kidney ammoniagenesis, thereby promoting uric acid stone formation (Daudon *et al.,* 2006). In case of insulin resistant patients the risk of kidney stones formation increases (Taylor *et al.,* 2005). The link between kidney stones and blood pressure (BP) is heterogeneous due to the differential effects of nephrolithiasis among sub groups of individuals. In overweight renal stone formers females the risk of hypertension is increased significantly by 69% while in men it was non-significant. The estimated difference in mean systolic and diastolic BP in stone formers with non-stone formers increase with body mass index in both genders, but was more pronounced in women (Gillen *et al.,* 2005).

2. MATERIALS AND METHOD

Case History

A 57 years old male patient visited OPD of Shifa ul Mulk Memorial Hospital, Hamdard University in September 2016 with lumbar pain. The interview regarding demographic data such as age, duration of disease, socioeconomic group, diet and living conditions were noted. The patient had a history of recurrent lower urinary tract infection, along with frequent urination and its urgency, difficulty in voiding and less urinary output. Physical examination revealed mild tenderness in left lumbar region. However, there was no family history of renal and vesicle calculi, but the patient was known case of diabetes and ischemic heart disease.

2.1. *Laboratory Investigations and Ultrasound*

The laboratory studies, included measurements of blood urea and creatinine using Microlab 300 (Merck Germany), Blood (3 ml) was collected through vasopunture in tubes containing anti-coagulant ethylene diamine tetraacetic acid (EDTA, 3.6 mg). In case of complete picture (CP) parameters such as hemoglobin, red blood cells, white blood cells (neutrophils, lymphocytes, eosinophil, monocytes and basophils) and platelet count using

Table 1a: Constituents of Polyherbal Unani Formulation NU-08

S.No.	Botanical and Unani name	Image	Temperament	Pharmacological properties	Part used	References
1.	*Cichorium intybus* L. (*Tukhm kasni*)		Cold and Dry (1st order)	Anti-diabetic, Diuretic, Resolvent,	Seed	Street *et al.,* (2013)
2.	*Cucumis melo* L. (*Tukhm e kharpaza*)		Cold and Moist (1st order)	Diuretic, Febrifuge, Nutritive	Seed	Ullah *et al.,* (2015)
3.	*Cucumis sativus* L. (*Tukhm e Khayaren*)		Warm (2nd order) Dry (1st order)	Demulcent, Nutrient, Cooling Diuretic	Seed	Sahu *et al.,* (2015)
4.	*Foeniculum vulgare* M. (*Badyan*)		Warm and Dry (2nd order)	Anti-inflammatory, Anti-coagulant, Antidote, Diuretic, Appetizer, Carminative	Seed	Rather *et al.,* (2015)
5.	*Melia azadarach* L. (*Neem*)		Warm and Dry (2nd order)	Analgesic, Antipyretic, Antiseptic, Anti-inflammatory, Blood purifier	Leaves	Khan *et al.,* (2011)
6.	*Peucedanum grande* C.B. (*Doku*)		Warm and Dry (3rd order)	Carminative, Diuretic, Resolvent	Seed	Maqbul *et al.,* (2017)

S.No.	Botanical and Unani name	Image	Temperament	Pharmacological properties	Part used	References
7.	*Sphaeranthus indicus* L. (*Gulmundi*)		Warm and Moist (2nd order)	Anti-inflammatory, Blood purifier, Antipyretic, Astringent, Styptic	Flower	Galani *et al.,* (2010)
8.	*Tephrosia purpurea* L. (*Sarphoka*)		Warm And Moist (1st order)	Antipyretic, Blood purifier, Diuretic	Root	Shenoy *et al.,* (2010)
9.	*Tribulus terrestris* L. (*Kharkask*)		Warm and Dry (1st order)	Diuretics, Anti-inflammatory	Seed	Ali *et al.,* (2003)

Amount of each plant = 3 g

Table 1b: Constituents of *Hab-e-Rasoot* (1 Tablet B.D.)

S.No.	Botanical and Unani name	Image	Temperament	Pharmacological activity	Part used	References
1.	*Berberis aristata* (*Rasoot*)		Cold (2nd order) and Dry (3rd order)	Anti-infective, Febrifuge	Rasoot	Sati *et al.,* (2015)
2.	*Commiphora wightii* (*Gugal*)		Warm and Dry (3rd order)	Diuretic, Resolvent, Emollient	Gum	Shen *et al.,* (2012)
3.	*Terminalia chebula* (*Halila Zard*)		Cold (1st order) and Dry (2nd Order)	Diuretic, Cardio-tonic, Purgative	Seed	Kannan *et al.,* (2010)

Nos. 1, 2, 3 herbal drugs are in specific combination to form *Hab-e-Rasoot*.

SYMBEX X.21 (Japan) were noted. The ultrasound of kidneys and urinary bladder (KUB) was also recorded using ELOKA SSD 500 (Japan).

2.2. *Treatment*

A Unani polyherbal coded drug NU-08 (Table 1a) was prescribed as a decoction of one sachet in a cup of boiling water twice a day (BD). Additionally, *Hab-e-Rasoot* (Table 1b) was also included as one tablet, BD.

3. RESULTS AND DISCUSSION

The patient complaint about dysuria, urgency and frequent urination along with less output of urine. Since, these were the most common clinical signs and symptoms of renal and vesicle calculi, therefore he was diagnosed as a patient of *Hisat-e-Kulia wa Masana* (urolithiasis). It was further confirmed by the laboratory investigation and ultrasound. On laboratory studies, blood urea was 38 mg/dl (range 10-50 mg/dl) and creatinine 0.8 mg/dl (range 0.6-1.5 mg/dl) were within normal range, while hemogram showed reactive neutophilic leukocytosis. Renal ultrasound revealed single calculus in left kidney measuring 0.4 cm as well as urinary bladder showed multiple calculi largest ranging from 0.7 and 0.8 cm, along with enlarged prostate (38.3 g) as compared to normal prostate (20 g).

After 1-week of treatment with polyherbal unani medicine (NU-08) and *Hab-e-Rasoot*, a slight improvement was noticeable and patient remarked that the pain, urgency and frequency of urine have been reduced, and hence advised to continue the treatment. Upon 2nd follow-up visit (after 1-week) a significant improvement was observed as patient was able to pass urine without any difficulty and lumbar pain also disappeared. On 3rd visit (Day-21) patient informed that one tiny and one large stone (~1.0 cm) (Fig. 1) was excreted in urine.

Fig. 1: Calculi measuring ~1 cm which is excreted in Urine of Patient

The Unani preparation used NU-08 contained nine medicinal plants (Table 1a) which have been reported to possess diverse pharmacological properties particularly diuretics, anti-inflammatory, anti-septic, resolvent and anti-pyretic. The medicinal plants were *Tribulus terrestris* (*Kharkhask*), an anti-inflammatory for the mucous membrane of urinary tract (Ali *et al.,* 2013). *Peucedanum grande* (*Doqu*), a diuretic (Makbul *et al.,* 2017). *Tephrosia purpurea* (*Sarphoka*) roots inhibit urinary tract infections and fever caused by urinary tract infection. *Cucumis sativus* (*Tukm-e-Kyareen*) and *Cucimus melo* (*Tukm-e-Kharbaza*) being diuretics with febrifuge properties resulted in excretion of calculus (Sahu *et al.,* 2015). *Foeniculum vulgare* (*Badyan*) have diuretic and anti-inflammatory properties (Rather *et al.,* 2015). *Cichorium intybus* (*Kasni*) is antidiabetic, diuretic and resolvent (Street *et al.,* 2013).

Hab-e-Rasoot consist of three medicinal plants as described in Table 1b. It was prescribed because patient was complaining of repeated urinary tract infection and after using, he was completely cured. This effect may be due to the presence of *Berberis aristata*

(*Rasoot*) which has antibacterial properties (Sati *et al.,* 2015).

It is concluded that Nu-08, Unani polyherbal medicine and *Hab-e-Rasoot* are effective, safe and economical against *Hisat-e-Kuliya wa Masana* (Renal and vesicle calculi). The availability of all the components of this Unani formulation is abundantly found in this region.

4. CONCLUSION

In traditional system herbal formulations could proved to be a far better lithotripic agent in various combinations against renal patients as compare to conventional drugs. Further research required in this regard in future.

Author's Contribution and Declaration

Umme Hani Ramzan: conducted the trial. Abdul Hannan: supervised the case study. The authors declared there is no conflict of interest.

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Cuscuta reflexa Roxb. and Its Pure Compounds-induced Micronuclei in Plant Cells and Downregulation of EGFR Expression in NCI-H460 Cell Line

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Abstract

Cuscuta reflexa Roxb. (Convolvulaceae) has medicinal applications used in treating wide range of diseases including cancer. The methanolic extract of *C. reflexa* Roxb. (MECR) grown on host *Nerium oleander* L.) and its pure compounds namely, odoroside H (1), neritaloside (2) and strospeside (3) were assessed for genotoxicity using mitotic meristematic root tip cells of *Allium* species (*A. cepa* and *A. sativum*) and meiotic pollen mother cells from *Tradescantia pallida* var *purpurea*. The effect of pure compounds were also evaluated in human non-small cell lung cancer cell line (NCI-H460) on tumor suppressor gene (p53) and epidermal growth factor receptor (EGFR) expression using agarose gel electrophoresis.

The MECR between 0.1 and 1 mg/ml was ineffective in mitotically dividing cells, whereas in meiotic pollen mother cells, higher concentrations (5-20 mg/ml) caused significant induction of micronuclei (MN). In both mitotic and meiotic cells, odoroside H and neritaloside induced significant rise in MN frequency, however, strospeside had no effect. The genotoxic potential in both cells was in the order of: (1) = (2) > MECR > (3).

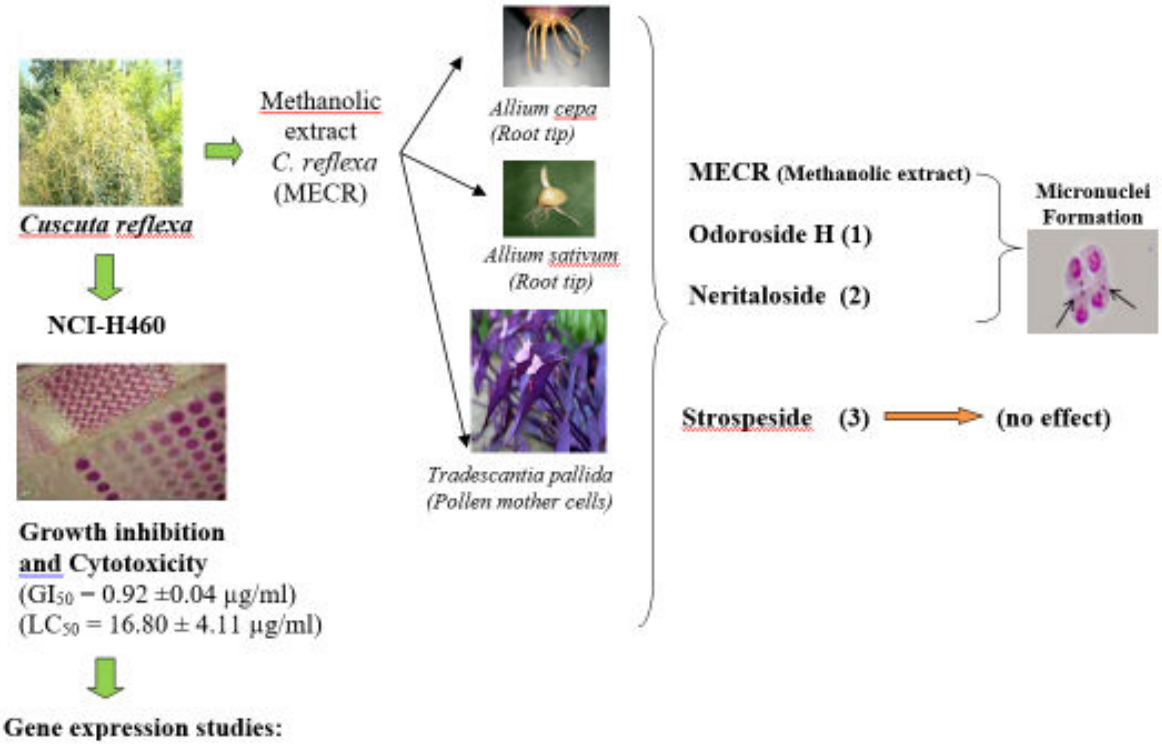
MECR and odoroside H (1) caused dose dependent growth inhibition in NCI-H460 cells with GI50 of 0.92 ± 0.04 µg/ml and of 0.08±0.01 µg/ml, respectively. The antiproliferative action of MECR (1 µg/ml), odoroside H (1) (0.14 µM), neritaloside (2) (0.16 µM) and strospeside (3) (0.5 µM) in comparison to control, significantly downregulated expression of epithelial growth factor receptor (EGFR) by two fold with no affect on p53 gene expression.

This study clearly demonstrates that MECR is genotoxic at higher doses and should be taken into account if used for medicinal purposes. The mitotic cells of *Allium* species were more sensitive (~2×) in MN formation than meiotic cells of *T. pallida pu*. Furthermore, MECR and pure compounds possesses anti-proliferative activity against NCI-H460 cells that might be mediated through EGFR-dependent but p53-independent pathways.

Keywords

Cuscuta reflexa Roxb., Genotoxicity, Micronuclei, Anti-proliferative, NCI-H460.

Graphical Abstract



1. INTRODUCTION

Cuscuta reflexa Roxb. commonly known as amarbel (meaning, immortal vine), belongs to Convolvulaceae (Morning glory) family. It is a leafless, rootless parasitic plant that depends on host plant for support and food supply (Ashwani *et al.,* 2012). It is parasitic on wide variety of plants such as *N. oleander* potatoes, alfalfa, flax, dahlia, trumpet vine, clover, lespedeza etc. In traditional medicinal system it is used against diseases of spleen, cancer, jaundice, headache, insanity, melancholy and fits (Ashwani *et al.,* 2012). Multiple pharmacological properties have been associated with it including anti-bacterial, anti-viral, anti-oxidant, anti-cancer, anti-inflammatory (Suresh *et al.,* 2011), psychopharmacological (Pal *et al.,* 2003), anti-spasmodic (Gilani and Aftab, 1992) and anti-hypertensive (Singh and Garg, 1973). *Tradescantia* Recently, antidepressant effect of *C. reflexa* Roxb. has been reported to be associated with quercetin-induced increase in neuronal serotonin and noradrenaline levels *via* inhibition of monoamine oxidases (Abbas *et al.,* 2016).

The phytochemical analysis of *C. reflexa* Roxb. grown on *N. oleander* revealed the presence of wide range of chemical constituents *viz*. amarbelin, b-sitosterol, cuscutin, coumarin, dulcitol, kaempferol, myricetin, oleanolic acid, quercetin and stigmasterol (Versiani, 2004). Despite its several pharmacological properties,

as mentioned above, there is no information regarding its genotoxic effects. It is of utmost importance to know the mutagenicity of traditionally used natural products which needs to be evaluated prior to its in-depth pharmacological investigations (Sannomiya *et al.,* 2007). The phytochemical along with pharmacological studies revealed that, cardenolide glycosides *viz* neritaloside, odoroside H and strospeside, possesses potent anti-cancer activity against different human cancer cell lines (Versiani, 2004; Versiani *et al.,* 2017).

In our recent studies, MECR, neritaloside and odoroside H, exhibited a dose-dependent reduction in root length, mitotic index (MI) accompanied by an increase in chromosomal aberrations (CAs), predominantly metaphase aberrant, sticky chromosome and spindle disturbances, therefore, these compounds deminstrated a stronger aneugenic effect as well as a weaker clastogenic effect in both *A. cepa* and *A. sativum* (Ali *et al.,* 2017). This prompted us to further investigate their effect on micronuclei formation.

Micronuclei (MN) popularly known as Howell-jolly bodies, are DNA-containing extranuclear bodies formed either after chromosomal breakages (clastogenic) or during mitotic stages of cell division leading to aneuploidy and referred as aneugenic in nature (Natarajan and Obe, 1982). These were identified and quantified as small independent stained bodies different from the main nucleus (Tolbert *et al.,* 1991, 1992). MN are also formed spontaneously, due to genetic instability or due to insults by common genotoxic agents, such as ionizing radiation, air pollutants, and chemicals including drugs causing an increase in their frequency and considered as a good index for classifying them as genotoxins (Neri *et al.,* 2003).

Allium due to its manageable number (2n=16) and large sized chromosomes, is considered to be most efficient and suitable system for genotoxic evaluation and identification of mutagenic substances and is equally suitable for evaluating complex mixtures and pure compounds with reliable accuracy (Leme and Marin-Morales, 2009). The genotoxicity in meiotic cells is determined using *Tradescantia* clone 4430 or 03. Trad-MN assay is a quick, simple and efficient system to monitor mutagens/ carcinogens. It is used for monitoring environmental genotoxicity and also mutagenicity (Guimaraes *et al.,* 2000) of drugs including natural products and herbal preparations by quantifying the appearance of micronuclei in highly sensitive germ cells of the *Tradescantia* species (Zhang *et al.,* 1999)**.** The increase in genetic damage in meiotic cells of *T. pallida* var. *purpurea* observed as MCN frequencies in tetrads of flower buds exposed to test agents (Cassanego *et al.,* 2014). When exposed to genotoxic and mutagenic agents, its reproductive cells (tetrads) exhibit nuclear fragments (MN) resulting from chromosomal breakage or loss, indicating genotoxic damages (Carneiro and Takayanagui, 2009; Mielli, 2009).

Non-small cell lung cancer (NSCLC) is the major cancer killer worldwide in both genders, accounting for >1.2 million deaths per year, emphasizing the need for more efficient therapeutic strategies (Jemal *et al.,* 2006). The major signaling pathways that could provide therapeutic targets comprise the following: a) Growth promoting pathways (Epidermal growth factor receptor/phosphatidylinositol 3-Kinase/Ras), b) Growth inhibitory pathways (STK11, p53/Rb/P14ARF), c) Apoptotic pathways (FasL/Bcl-2/Bax/Fas), d) Immortalisation and e) DNA repair genes (Brambilla and Gazdar, 2009). Transcription of *p53* is at minimal level in normal tissue which is elevated by genotoxic agents (Sun *et al.,* 1995). Epidermal growth factor receptor (EGFR) is a member of

the receptor tyrosine kinase (TK) family, which includes the erbB family. TKs control signaling pathways and regulate critical cellular activities (Blume Jensen and Hunter, 2001). It is commonly over expressed (40%-80%) in cancer cells like NSCLC and has been linked to its pathogenesis (Scagliotti *et al.,* 2004).

2. MATERIALS AND METHOD

2.1. *Chemicals*

Bleomycin hydrochloride (Nippon, Japan), ethanol (Tedia, USA.), gelatin (Fluka AG, Switzerland), glacial acetic acid (Labscan, Thailand), orcein (W.S. Simpson, London), sodium azide (Ogawa Seiki, Japan), doxorubicin HCl (Korea United Pharm. Inc. and MP Biochemicals, USA), vinblastine sulphate (Pharmedia Laboratories, Pakistan). Dimethyl sulfoxide (DMSO), doxorubicin and all other chemicals of highest purity were used (Sigma, USA).

2.2. *C. reflexa Roxb. Plant Collection*

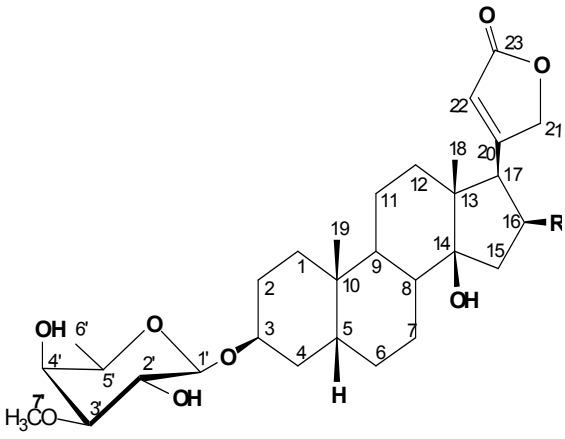
C. reflexa Roxb. growing as a parasite over host plant, *Nerium oleander* L. growing wild at final stage (Fig. 1) was collected from Karachi University and authenticated by Dr. Rubina Dawar Department of Botany, University of Karachi, Pakistan with a voucher specimen (No. 66855 KUH) and submitted in the herbarium of the same department. *Allium cepa* L. and *Allium sativum* L. were purchased from vegetable market, Karachi.

2.2.1. *Preparation of Methanolic Extract of C. reflexa Roxb. (MECR)*

Fresh, undried, uncrushed plant of *C. reflexa* Roxb. (3.5 kg) was extracted with methanol at room temperature for four times. The combined extracts were freed of the solvent in *vacuo* to afford thick residue (31.65 g), referred as methanolic extract of *C. reflexa* Roxb. (MECR) and partitioned between aqueous, and ethyl acetate phases. The yellow powder (2.31 gm) was obtained from ethyl acetate phase by using solvent separation techniques which afforded neritaloside (105 mg), odoroside H (90 mg) and strospeside (57 mg) by subjecting

Fig, 1: *Cuscuta reflexa* Roxb. (Amar bel)

to vacuum liquid chromatography (VLC) followed by flash column chromatography. The structures of these compounds (Fig. 2) were elucidated and identified earlier through extensive spectroscopic methods (Versiani, 2004).



	R
Odoroside H (1)	H
Neritaloside (2)	$OCOCH_3$
Strospeside (3)	OH

Fig. 2: Structures of cardenolides (**1-3**)

2.2.2. *HPLC Analysis*

The HPLC system equipped with four LPG-3400A reciprocating pumps and variable wave length ((Dionex, VQDD 3100Thermo Scientific, USA), UV-VIS detector and injector with loop size of 20 ml (Rheodyne Model 8125) was used and reverse phase chromatography was carried out at 25oC (Figs. 3a and b). Samples, extract (0.68 mg/ml) and odoroside H, neritaloside and strospeside (0.46 mg/ml) prepared in methanol, were filtered through an ultra membrane filter (pore size 0.45 mm, Millipore, DURAPORE, Ireland) prior to injection in the sample loop.

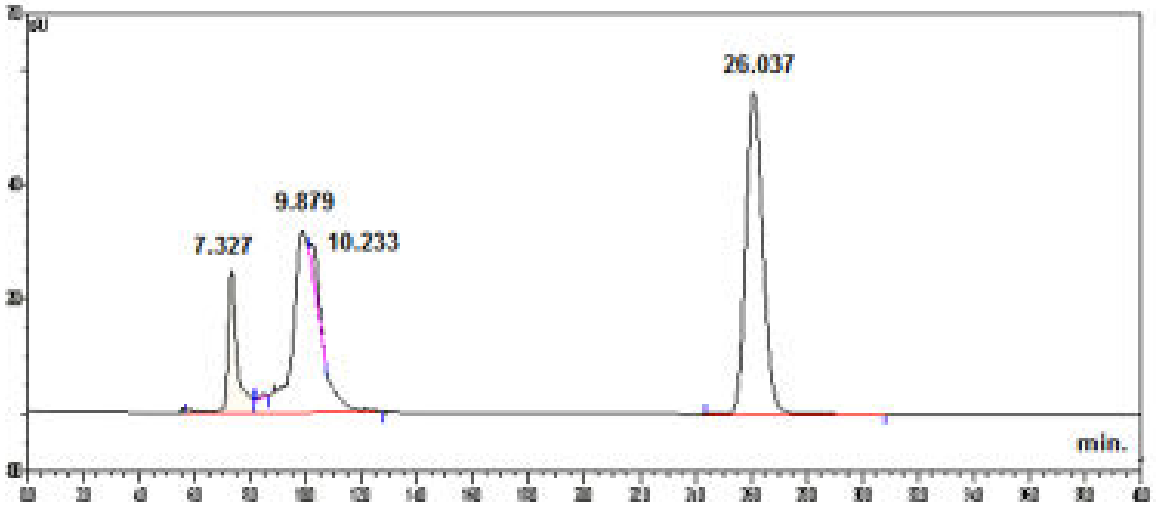
2.3. *A. cepa L. and A. sativum L. Assays*

Both *Allium* species were purchased from local market and dried scales were removed without damaging root primordial. The *A. cepa*, onion bulbs (5 kg) (n=5) and *A. sativum* garlic cloves (1 kg) (n=3) were made to germinate in tap water to obtain roots up to 3 cm and 2 cm, respectively followed by treatment with different test agents for 48 h. Owing to limited amounts of pure compounds their genotoxicity was assessed using garlic bulbs. After treatment, the root tips were fixed in ethanol:acetic acid (3:1) for 24 h followed by hydrolysis with 1 N HCl (5-15 min). After rinsing with distilled water twice, the root tips were stained with aceto-orcein (2%) for a period of 30 min., placed on to a glass slide containing two drops of glacial acetic acid (45%) and individually teased with a needle. A cover slip was carefully placed to avoid air bubbles and squashed gently (Sehgal *et al.,* 2006). The induction of MN formation was assessed as described earlier (Fiskesjo, 1985; Shaymurat *et al.,* 2011) with slight modifications. To observe MN induction, about 300-400 cells/slide (onion) and 1000 cells/slide (garlic) were counted.

2.4. *Tradescantia Micronucleus (TRAD-MN) Bioassay*

T. pallida pu., was cultivated and maintained during 2011 in the open field nursery of International Center for Chemical and Biological Sciences, University of Karachi, Karachi, Pakistan. The Trad-MN was conducted as described earlier (Ma, 1981), with slight modifications. The plant cuttings with young inflorescence (5-8 cm) were removed from the plant and were cut in a slanting shape (to provide better liquid absorption). About 12-15 cuttings were placed in glass jars containing testing solutions (100 ml) for 24 h and placed for recovery period (D/W, 100ml) for further 24 h.

a)



b)

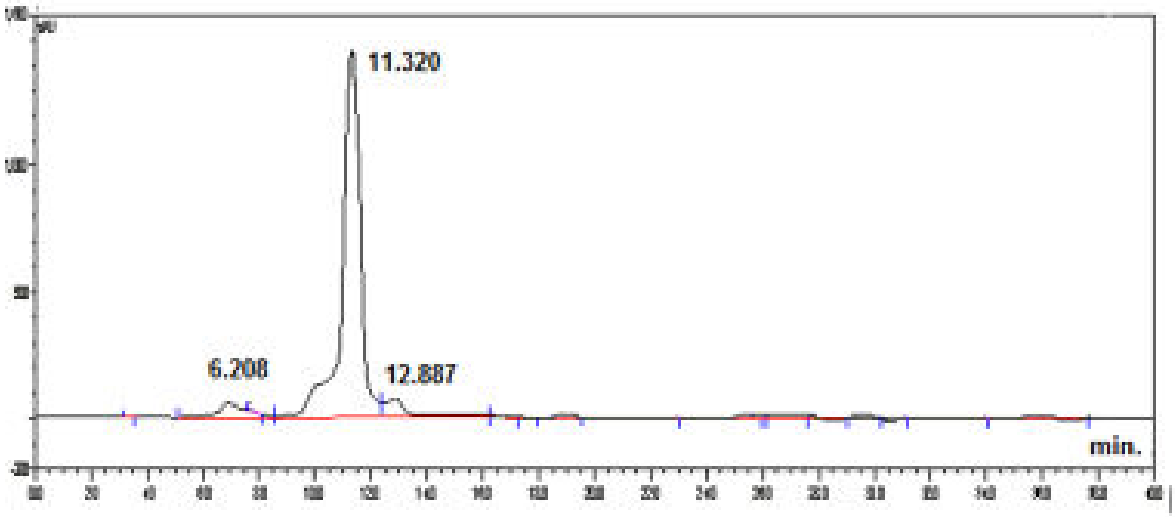


Fig. 3: HPLC chromatogram of methanolic extract of *Cuscuta reflexa* Roxb. and odoroside H

a) Methanolic extract of *C. reflexa* Roxb. (0.68 mg /ml) and b) Odoroside H (**1**) (0.46 mg/ml)

Chromatographic conditions: Lichrocart C-18 column, mobile phase acetonitrile/water (8.5:1.5) with flow rate 0.20 ml/ min. and detected at 254 nm.

The inflorescences were fixed in acetic acid:ethyl alcohol (1:3) fixative solution for 24 h and stored in ethanol (70%). For slide preparation anthers were separated from the flower bud and placed onto a glass slide in a drop of aceto-orcein stain (2-5 min), crushed gently to release meiotic pollen mother cells (PMC) undergoing tetrad stage. The debris was carefully removed and cover glass was placed on it. This temporary slide was observed and scored for stained nuclei, micronuclei under Nikon microscope 400× magnification. The percentage of MN per 100 tetrad was calculated:

$$\text{MN in tetrad (\%)} = \frac{\text{MN count}}{\text{Tetrad count}} \times 100$$

2.5. *Anticancer Sulforhodamine B (SRB) Assay*

The human non-small cell lung cancer cell line, (NCI-H460) was employed for the anticancer evaluation of test agents using sulphorhodamine-B (SRB) assay as described earlier (Skehan *et al.,* 1990; Qamar *et al.,* 2010). Briefly, the cells (7,500 cells/100 µl/well) in 96-well plate were incubated for 24 h at 37°C in a humidified 5% CO_2 incubator and treated with 100 µl of different concentrations of MECR (0.1, 1, 10, 100, 250 µg/ml), odoroside H and neritaloside (0.001, 0.01, 0.1, 1, 10 µM), strospeside (0.01, 0.1, 0.2, 0.3, 0.5 µM), doxorubicin and vinblastine (0.01, 0.1, 1, 10, 100 µM). After 48 h of incubation period, ice-cold trichloroacetic acid (TCA: 50 µl/well, 50%, wt./vol.) was added for 30 min., washed (5×) with distilled water and dried overnight. The SRB solution (100 µl/well, 0.4%, wt./vol. in 1% acetic acid) was added and after 30 minutes the unbound stain was removed by rinsing four times with acetic acid (1%) and left for air drying overnight. The protein-bound stain was solubilized with tris-base solution (100 µl/well, 10 mM) and shaken for 5 minutes at room temperature on a plate shaker. The absorbance was measured at 515 nm using 96-well plate reader. The absorbance was used to calculate the growth inhibition (GI50), total growth inhibition (TGI) and cytotoxicity (LC50) values using appropriate controls.

2.6. *Effect of MECR and Its Pure Compounds on p53 and EGFR Expression*

Cells (NCI-H460, 2×106 cells) were seeded in flasks (25 cm^2) and incubated in a CO2 incubator at 37C for 24 h to form the monolayer. Doxorubicin (0.1 µM), plant extract MECR (1 µg/ml), odoroside H (0.14 µM), neritaloside (0.16 µM) and strospeside (0.5 µM) were added. After 48 h of treatment, cells were harvested and total RNA was extracted using the TRIzol reagent (Life Technologies) according to the manufacturer's instructions. The concentration of RNA was determined spectrophotometrically (280 nm) using of total RNA (2 µl) to quantitate on the nanodrop and nuclease free water (2 µl) was used as blank. For cDNA synthesis, total RNA (1 µg) was mixed with 50 µM of Oligo$(dT)_{20}$ primer (1 µl), 10mM dNTP (1 µl), and nuclease free water up to 8 µl using SuperScript III-first strand synthesis kit (18080-300, Invitrogen Life Technology, USA). The cDNA was amplified by using Invitrogen PCR kit. The reaction mixture (50 µl) was prepared by mixing: PCR master mix (46 µl), cDNA (2 µl) and forward and reverse primers (200 nM, 2 µl) for p53 and EGFR genes and placed in a PCR thermal cycler for cDNA denaturation (94°C for 1 min), followed by 30 cycles of amplification with following conditions: denaturation (94°C for 1 min), annealing (59°C for 1 min) and extension (72 °C for 1 min) and finally extension at 72 °C for 10 min. The reaction mixture was subjected

to electrophoresis in agarose gel (1%) stained with ethidium bromide. The bands were visualized (UV) and quantified using gel documentation and compared with respective control.

2.7. *Statistical Analysis*

Statistical analysis was performed using SPSS 12 software by analysis of variance (ANOVA) followed by post hoc test with least significant difference (LSD) (*p<0.05; **p< 0.01; ***p<0.001) and Duncan's multiple range tests p value of <0.05 was considered statistically significant.

3. RESULTS AND DISCUSSION

C. reflexa Roxb. has been used in traditional medicine for treatment of various ailments; however, its genotoxic information is unclear. In our previous study, MECR (100-5000 µg/ml) induced a dose dependent reduction in root length (6.24%-92.19%) in *A. cepa,* whereas, 4x more reduction was observed in *A. sativum* (Fig. 5). Mitotic index was also reduced (5.4%-0.5% and 4.78%-1.11%) in *A. cepa* and *A. sativum,* respectively. The genotoxic potential of MECR was further supported by the induction of various types of chromosomal aberrations (Aberrant metaphase, stickiness, bridges and fragments) in the root tips of Allium species (Ali *et al.,* 2017). Keeping this in mind, MECR and pure compounds derived from it namely, odoroside H, neritaloside and strospeside were evaluated for their genotoxic effects focusing on MN formation using two different plant cells: ***1***) mitotic cells *A. cepa* and *A. sativum* and ***2***) meiotic cells *Tradescantia pallida pu*.

Due to ease of detection, micronucleus is popularly used as a biomarker of chromosomal defects induced by genotoxins (Luzhna *et al.,* 2013). The induction of micronuclei in any organism is the manifestation of chromosomal breakage and the disturbance of the mitotic process due to spindle abnormalities, indicating true mutation effect (Auer, 1962). In *Allium* root tip cells, the induction of micronuclei in the presence of MECR was dose dependent and its frequency increased (0.9%) as compared to control (0.15%). In both *Allium* species, MECR (100 µg/ml) induced MN formation that was 6x greater as compared to control (0.15%). Among the standard drugs used bleomycin (50 µM) caused maximum increase (~27×) in the frequency (2.72%) of MN. The pure compounds odoroside H and neritaloside (10 µM) caused significant induction of MN (Table 1a).

The micronuclei in the meiotic cells (tetrads) of *T. pallida pu.* were identified to be present outside the nucleus Fig. 4 (a-h) and were relatively 1/3rd to 1/5th of its size. MECR induced a dose-dependent rise in the frequency of micronuclei. At low concentration (5 mg/ml) micronuclei induction was similar to control (1.60%). However, at higher concentration (10 and 20 mg/ml) the micronuclei were increased, and at highest dose tested (30 mg/ml) and tetrads were not observable. Pure compounds odoroside H and neritaloside at 10 µM also caused significant induction of MN (~5× greater) as compared to control (Table 1b). The spontaneous frequency of MN in *T. pallida pu.* (control) was only 1.45% almost similar to that reported earlier (de Oliveira *et al.,* 2007). However, in the presence of MECR, a dose dependent rise (~4%-13%) by ~3×-8× at 5-20 mg/ml, followed by absence of tetrads at higher doses (30 mg/ml) clearly suggest that plant extract is genotoxic and its magnitude of damage is concentration dependent. Odoroside H and neritaloside showed significant MN formation (2×-5×) in mitotic cells, while in meiotic cells only high concentration (10 µM) significantly induced MN formation (4.5×).

Table 1a: Effect of Methanolic Extract of *C. reflexa* Roxb., Odoroside H, Neritaloside, Strospeside, Sodium Azide, Bleomycin and Vinblastine on Micronuclei Formation in Mitotic Cells of *A. cepa* and *A. sativum*

Test substances	Mitotic cells	
MECR (mg/ml)	***A. cepa***	***A. sativum***
0.01	0.59^{3}±0.18	
0.1	0.96^{2}±0.13	$0.78^{1,2}$±0.03
1	0.56^{3}±-0.09	$0.51^{2,3}$±0.06
5	0.12^{5}±0.03	
Pure compounds (µM)		
Odoroside H 1	nd	
10	nd	0.72^{1} ± 0.04
Neritaloside 1	nd	$0.26^{4,5}$±0.04
10	nd	0.65^{1}±0.06
Strospeside 1	nd	0.10^{6}±0.02
10	nd	$0.17^{5,6}$±0.03
Standard drugs (µg/ml)		
Sodium azide 1	0.61^{3}±0.19	0.49^{3}±0.09
50	Cell death	Cell death
Bleomycin (50)	2.72^{1} ± 0.50	
Vinblastine (15)	0.84^{2}± 0.04	
Control	$0.17^{4,5}$±0.04	0.13^{6}±0.01

Table 1b: Effect of Methanolic Extract of *C. reflexa* Roxb., Odoroside H, Neritaloside, Strospeside, Sodium Azide, Bleomycin and Vinblastine on Micronuclei Formation in Meiotic cells of *T. pallida*

Test Substances	Meiotic cells
MECR(mg/ml)	***T. pallida***
0.01	0.82^{6}±0.29
0.1	1.64^{6}±0.07
1	$3.42^{5,6}$±0.55
5	4.33^{5}±0.06
10	$7.60^{3,4}$±0.05
20	$11.83^{1,2}$±1.11
Pure compounds (µM)	
Odoroside H	
1	$2.05^{5,6}$±0.36
10	$7.78^{3,4}$±0.78
Neritaloside	
1	$2.17^{5,6}$±0.73
10	6.73^{4}±0.15
Strospeside	
1	0.94^{6} ± 0.20
10	1.66^{6} ± 0.24
Standard drugs (µg/ml)	
Sodium azide	
1	
50	$2.89^{5,6}$ ± 0.628.65^{3,4} ± 0.58
Bleomycin **(50)**	13.42^{1}±1.65
Vinblastine **(15)**	$10.03^{2,3}$±0.69
Control	1.60^{6}±0.20

Distilled water and vehicle control (2% DMSO) gave similar results therefore the data was pooled and referred as control.

Methanolic extract of *C. reflexa* = MECR

Total number of mitotic cells observed, in control and for each treatment = 9000

Total number of tetrads counted = 1100-2000 in 3 independent experiments

Each value represents percent mean ± SEM of micronuclei

Not determined = nd

The dissimilar numerical superscripts (1-6) in columns are significantly ($P<0.05$) different from each other, whereas, homogenous means are represented by similar numerical superscripts

Pure compound includes: Odoroside H (**1**), Neritaloside (**2**) and Strospeside (**3**)

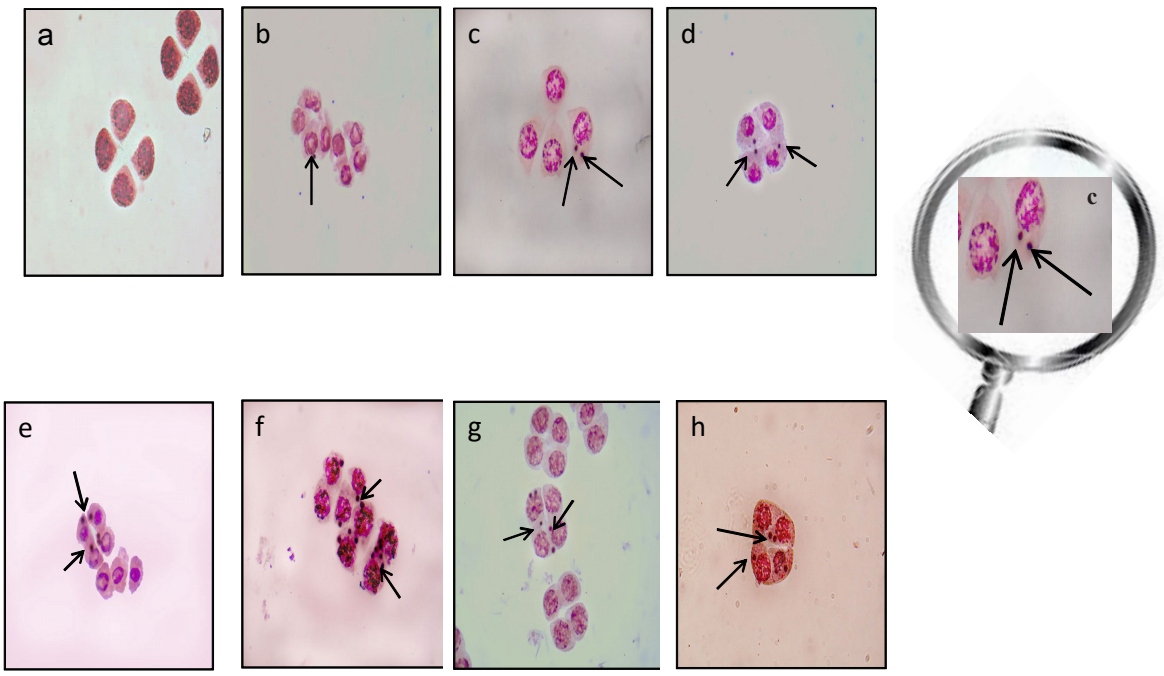


Fig. 4: Meiotic products of *T. pallida* in the absence or presence of methanolic extract of *C. reflexa* Roxb.

Meiotic products of *T. pallida* showing tetrads in: a) Control, b) DMSO (2%) or in the presence of different concentrations of methanolic extract of *C. reflexa* Roxb., c) 5 mg/ml, d) 10 mg/ml, e) 20 mg/ml or anti-cancer drugs, f) Bleomycin (50 µg/ml) and g) vinblastine (15 µg/ml) and h) sodium azide (50 µg/ml).

Micronuclei are identified by arrows.

MECR induced MN formation in both mitotic and meiotic systems, while among pure compounds: odoroside H and neritaloside were genotoxic in both systems whereas, strospeside was ineffective indicating that former two compounds are genotoxic. These differences may be due to the presence of hydroxyl (–OH) group at position 16 in strospeside which makes it less genotoxic. However, the presence of acetyl ($COCH_3$) group at position 16 in neritaloside and its absence in odoroside H apparently has no role in the genotoxic effects as reflected by similar effects in the aforementioned parameters. Recently, glioma-associated oncogene (GLI) transcriptional inhibition (IC_{50} in µM) has been reported for cardenolides, in which the importance and the influence of substitution at C-16 of the steroidal skeleton have also been recognized (Arai *et al.,* 2011).

Nevertheless, in our study both *Allium* species demonstrated that, mitotically dividing cells were more sensitive to genotoxic effect than meiotic cells of *T. pallida pu.* These

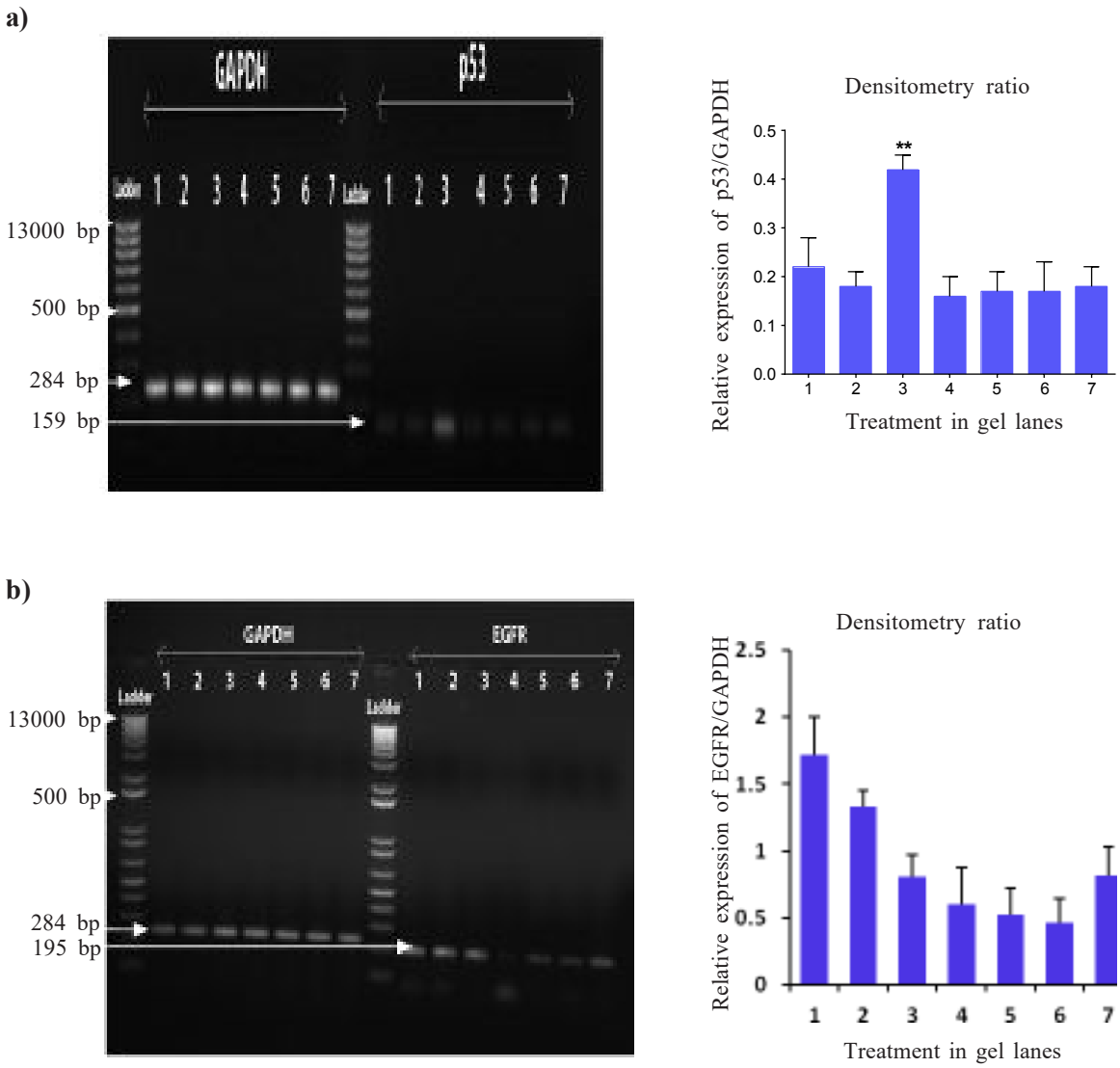


Fig. 5: The effect of methanolic extract of *C. reflexa* Roxb., odoroside H, strospeside and neritaloside on p53 and EGFR gene expression in NCI-H460 cells

The gene expression of a) p53 and b) EGFR in NCI-H460 cells was determined *via* agarose gel electrophoresis.

Agarose gel lanes are represented as: 1) Control, 2) vehicle control (DMSO 0.1%), 3) doxorubicin, 4) MECR, 5) odoroside H, 6) neritaloside and 7) and strospeside.

Base pair = bp

DNA ladder = 100-13000 bp

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control appearing as a single band of 284 bp

Analysis of variance (ANOVA) followed by post hoc test with least significant difference (LSD). *p<0.05; **p<0.01 and ***p<0.001 was considered statistically significant.

findings are contradictory as formation of micronuclei in meiotic tetrads were more sensitive than mitotic (root tip) cells, after treatment with metals (Misik *et al.,* 2011). This discrepancy may be due to the nature of genotoxins used as their concentration and availability into the cell are pre-requisite for inducing genotoxicity.

The chromatogram of MECR exhibited one major peak with a slight shoulder at retention time of ~10.0-11.5 minutes which coincided with the peak of cardenolides (odoroside H, neritaloside and strospeside, Figs. 3a and b). The percentage of odoroside H, neritaloside and strospeside in extract (1.136%) was calculated as follows:

> Peak area of sample/peak area of standard × weight of standard/dilution of standard × dilution of sample/weight of sample × 100.
>
> 18.255/1086.815 × 0.46 mg/1.0 ml × 1.0 ml/0.68 mg × 100 = 1.136

It has been observed that genotoxicity may appear due to the disturbance of oxidative/ antioxidative equilibrium implying that its severity is related to anti-oxidative capacity of compounds (de Souza Lima *et al.,* 2009). In our study, MECR demonstrated genotoxicity at high concentrations possibly due to the increased level of anti-oxidants such as kaempferol, myricetin, oleanolic acid and quercetin residing in it (Hollman *et al.,* 1997; Vinson *et al.,* 1995; Larson, 1988) and possibly causing DNA damages as reflected by a significant increase in the number of micronuclei as compared to control. Most recently, well established anti-oxidants, resveratrol, genistein and baicalein induced DNA damages leading to cell death without causing mutagenesis (Jennifer *et al.,* 2012) and hence has a potential to be used in cancer chemotherapy.

Natural plant products have been widely used in controlling cancer with fewer or no side effects. Recently, a flavonoid, patuletin from *Tagetes patula* flower has been reported to possess cytotoxic and growth inhibitory activities preferably towards cervical cancer (Kashif *et al.,* 2015) due to its antioxidant activity. MECR (1-250 µg/ml) demonstrated significant ($P<0.001$) dose dependent growth inhibitory/ cytotoxic activity (+51% to –82%). The corresponding GI_{50} (~1 µg/ml), cytostatic effect (TGI at 4.10 µg/ml) and the cytotoxic effect, LC_{50} (16.80 µg/ml) are presented in Table 2. The odoroside H (0.1-10 µM) showed significant ($p<0.001$) dose dependent growth inhibitory/ cytotoxic activity (+44% to –80%) with GI_{50} of 0.14 µM. Neritaloside at highest doses (1-10 µM) showed profound cytotoxicity (–10 to –80%) with GI_{50} of 0.16 µM. Whereas, strospeside was least active, with growth inhibitory activity (73% and 36.9%) at 0.3 and 0.5 µM, respectively with GI_{50} of 0.41 µM. The TGI (~0.62 µM) for odoroside and neritaloside was similar. However, LC_{50} for odoroside and neritaloside was (5.57 and 3.38 µM respectively). The TGI and LC_{50} for strospeside could not be determined (Table 3). Anticancer drugs bleomycin and vinblastine induced micronuclei formation in astrocyte micronucleus assay (Miyakoshi *et al.,* 2012) and cytokinesis block micronucleus assay (Verheyen *et al.,* 2003) respectively, but there is no report of their effects on *Allium species* and *T. pallida pu.* Both these drugs (bleomycin and vinblastine) induced micronuclei formation in both test with higher frequency in meiotic cells, implying their selectivity towards gametogenesis.

Andrographis nallamalayana (AN) showed anti-cancer activity on skin melanoma cancer cell lines (A375 and B16F10) through increased

expression of p53 (Purushotham *et al.,* 2016). Graviola fruit extract markedly downregulated EGFR gene expression and inhibited the growth of breast cancer cells (Dai *et al.,* 2016). In the present study an attempt has been made to explore the effect of MECR, odoroside H, neritaloside and strospeside on p53 and EGFR expression. In all the experiment, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control that appeared as a single band of 284 bp and thereby validated the experimental protocol. The p53 expression in control (RPMI), vehicle control (DMSO, 0.1%), MECR, odoroside H, neritaloside and strospeside were similar with densitometric ratio of ~0.24, indicating that p53 expression remains unchanged. However, a significant ($p<0.05$) upregulation (1.44×) of p53 was exhibited in the presence of positive control doxorubicin as compared to control **(**Fig. 5a).

The EGFR expression in control and vehicle control (densitometric ratio = 1.71±0.29) was similar (Fig. 5b). In the presence of MECR, odoroside H and neritaloside a significant ($p<0.01$) downregulation (~2.56×) of similar magnitude was observed whereas, strospeside showed lowest level of downregulation (1.62×) of EGFR as compared to control. Doxorubicin also demonstrated significant ($p<0.05$) downregulation (2.1×) of EGFR as compared to control which was lower (1.2×) than MECR and pure compounds.

Aqueous extract of *C. reflexa* Roxb. has been reported earlier to up regulate pro-apoptotic factor p53 in hepatocellular carcinoma (Hep3B) cells (Suresh *et al.,* 2011). However, our preliminary studies showed that the MECR, odoroside H, neritaloside and strospeside were ineffective towards p53 expression in non-small cell lung cancer cell line (NCI-H460). This may be due to the differences in the types of the cancer cells used or due to differences in the chemical constituents residing in aqueous extract that are absent in methanolic extract and requires further investigation. Earlier, EGFR expression in human nasopharyngeal carcinoma (NPC-TW02) and epidermoid carcinoma (A431) cells (Huang et al., 2007) was inhibited by a highly purified fraction (LC-X) from ethanol extract of *Livistona chinensis* R. Brown, seeds. Likewise, we report for the first time that methanolic extract of *C. reflexa* Roxb. and pure compounds *viz* odoroside H, neritaloside and strospeside derived from it also significantly downregulated EGFR expression and hence, this potential could be explored further against lung cancer using *in vitro* and *in vivo* studies. It is well established that over expression of EGFR in many tumors including NSCLC enhances tumor cell proliferation, angiogenesis, invasion and metastasis (Shen *et al.,* 2012). This makes EGFR as a possible target for NSCLC treatment as reflected by its successful use as monoclonal antibodies and as tyrosine kinase inhibitors (Doebele *et al.,* 2010).

In conclusion, *C. reflexa* Roxb., odoroside H, strospeside and neritaloside reported for the first time that they are genotoxic in both mitotic and meiotic cells, however, mitoic cells of *Allium* species were more sensitive than meiotic cells. This genotoxic effect is more likely due to the presence of equipotent odoroside H (**1**) and neritaloside (**3**). The induction of MN suggests that *C. reflexa* Roxb. contained constituents that are clastogenic/ or spindle fibres inhibitors.

Further, MECR and the pure compounds were also cytotoxic and anti-proliferative in NCI-H460 cells possibly, due to down regulation of EGFR and which was independent of p53 expression. These observations may serve as a reference for further investigations on molecular mechanisms involved in the genotoxicity of *C. reflexa* Roxb. for its safe and proper utilization.

Conflict of Interest

There are no conflicts of interest.

Acknowledgments

Dr. Nadia Khan, Department of Genetics, University of Karachi, Pakistan helped in statistical analysis.

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An Evidence Based Assessment of Intermediate State of the Body by Using Scientific Approach of Modern and Conventional Knowledge

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Abstract

There are two states of the human body either health or disease which may co-exist, however, there is an intermediate state between extremes of absolute health and total disease with variation in severity of illness. This core concept of Unani medicine regarding states of the body (*Ahwal-e-Badan*) was initiated by Jalinoos (Galen) who introduced intermediate state and explained its causes further more notion of intermediate state expanded by Ibn Sina who delineated the states of the body ranging from health to disease. According to him the stages of health and disease are radiant health, not absolute health, neither healthy nor diseased, potential illness, slight ill health and absolute disease. The idea of these states of the body now recognized globally. According to World Health Organization (WHO) only 5% of world population is healthy, 20% have various diseases and 75% are living in "Sub Healthy State" or "Gray State of Health". The objectives of this study was to re-defining intermediate state, reconsidering the signs and symptoms, set the laboratory investigations, relating it to the ancient theory of temperament and to established preventive measures. Healthy subjects male (n=13) and females (n=17) between 19-26 years of age were interviewed and examined by random sampling method, using a validated questionnaire which included complete history, general physical and systemic examination, body mass index (BMI) calculation and temperamental assessment. Biochemical analyses of complete blood count and erythrocytes sedimentation rate, lipid profile, liver function test and fasting blood glucose were performed. Temperature (°F), pulse (beats/min) blood pressure (mm Hg), are respiratory rate were measured following standard procedures. The percentages of normal and low BMI were 33.3% whereas high and obese were 16.7%. In both genders, there was a positive correlation between (BMI) and laboratory investigation's remarks and advises. This study revealed the concept of Unani Medicine that, besides the states of health and disease there is also various intermediate state in which one is neither totally sick nor healthy. The role of six essential factors such as *Hawa-e-Muheet* (Atmospheric air), *Makool wa Mashroob* (Food and drinks), *Harkat wa Sukoon-e-Badani* (Physical activity and response), *Harkat wa Sukoon-e-Nafsani* (Mental activity and response), *Naum wa Yaqza* (Sleep and wakefulness), *Ehtibas wa Istifragh* (Retention and elimination) is much more than just the mere avoidance of disease.

Keywords

Intermediate state of the body, I State, Health, Disease, Body Mass Index, Clinical parameters.

1. INTRODUCTION

According to World Health Organization (WHO) 'state between health and disease' or 'Gray state of health' is defined as "when all necessary physical and chemical indexes are negative using various diagnostic tools, apparently normal however, the person experiences discomfort and even pain." (WHO, 1948; 1946, Callahan, 1973; Jadad and O'Grady, 2008). From this definition, if there are signs of problems in the body, or some inadequacy in emotions, social ability, flexibility and attitude towards life, then the person is unhealthy. Health is measured as a holistic condition.

According to the WHO, a large population approximately 75% are actually living in the sub-healthy condition most likely due to increased social pressures and only 5% of the people are healthy in accordance with the healthy standard of WHO while 20% are suffering from various diseases. (Ottoson, 2000).

The intermediate state between health and disease has a long history as the ideology generated by the Jalinoos (Galen). However, in the middle ages it was argued by many school of thoughts with different opinions but Latin concept of "*Neutrum*" or "Neutralitas" along with the question of existence of such a state and the utility as *'sine qua non'* for physician to acknowledge was more popular (Van der lugt, 2011; Pesenti, 2000).

According to Jalinoos (Galen) "the neutral (*oudéteron*, translated as *neutrum* in Latin) can be understood in three different ways: first *qua* not participating in any of the opposites, second *qua* participating in both opposites and third *qua* participating now in this one and then in that one. The second *qua* participating in each of the two extremes equally, or participating more in one of the two (*Tegni* Ib). (Joutsivuo, 1999).

The central problem for medieval physicians was not to recognize the intermediary state clinically or to devise specific treatments for neutral bodies. This does not mean, however, that medical practice was irrelevant to medieval debates about the neutral state (Boudon and Galen, 2000).

1.1. *Causes of Intermediate State of the Body*

In accordance with Jalinoo's (Galen's) description Causes of Intermediate state are age related (infants or old aged), congenital or acquired configurational disorders and humoral/ temperamental disturbances associated with changes or variations in lifestyle governing factors (*Asbab-e-Sitta Zarooriya*) such as: *Hawa-e-Muheet* (Atmospheric air), *Makool wa Mashroob* (Food and drinks), *Harkat wa Sukoon-e-Badani* (Physical activity and response), *Harkat wa Sukoon-e-Nafsani* (Mental activity and response), *Naum wa Yaqza* (Sleep and wakefulness), *Ehtibas wa Istifragh* (Retention and elimination) (Gruner, 1973).

1.2. *Signs and Symptoms of Intermediate State of the Body*

Most of the signs and symptoms of intermediate state also come from the symptoms of humoral imbalance (*Alamat-e-Ghalba-e-Akhlat*). Some of them are given in Table 1 (Ahmad, 1980).

2. AIMS AND OBJECTIVES

The objectives of this study was re-defining intermediate state, determine the signs and symptoms of this state on the basis of ancient theory of humoral/temperamental imbalance (*Su'e Akhlat/Su'e Mizaj*), and set the laboratory investigations for the diagnosis and to establish preventive measures for restoring complete healthy state.

Table 1: Signs and Symptoms of Imbalances of the Humors (*Alamat-e-Ghalba-e-Akhlat*)

	Evidences	Blood (*Dam*)	Phlegm (*Balgham*)	Choler (*Safra*)	Black bile (*Sauda*)
General Features	General physique	Good	Effeminate, bones, tendons, joints well covered	Joints large	Emaciated
	Complexion	Reddy	Unduly pale.	Yellow tinge in skin and conjunctiva	Dusky, whole body seems dark and hairy
	Feel of body and state of the skin	Flesh Firm, Redness on rubbing, Furuncles	Soft and Cool	Dry and rough	Flesh hard skin rough, liable to dark eruptions and intractable ulcers
	Hairs	Normal Brownish	Absent on trunk	Hairy	Hairy
	Surface veins	Full, not prominent	Constricted	Thick and hard	prominent
Vegetative Faculties	Mouth/Tongue	Liability to pustules, unusual sweetness in mouth, Red tongue	Abundant sticky saliva, unusual sweetness in mouth	Bitter taste, Rough and dry	Our taste, less and thick saliva
	Pulse	Large, fast, full and strong	Soft tends to be slow and infrequent	Rapid	Small, slow and hard
	Urine	Reddish	White	Yellowish Brown	Dark colored or black, dense
	Faeces	Reddish brown normal in consistency	Soft stool, tendency to diarrhea	Tendency to diarrhea and dysentery	Usually constipation

	Evidences	Blood (*Dam*)	Phlegm (*Balgham*)	Choleric (*Safra*)	Black bile (*Sauda*)
Sensitive Faculties	Appetite	Increased	Decreased	Increased	Depraved, faulty longings
	Thirst	Increased	Diminish or absent esp. in std peoples	Increased.	Decreased
	Muscular tone	Weariness not accounted for by exertion	Flaccidity of limbs.	increased	Flabbiness
	Dreams	Sees red things, blood coming out of the body, of swimming in blood and the like	Sees waters, rivers, snow, rain, cold, thunder	Sees fires, yellow hags, objects appear yellow, a conflagration, hot bath hot sun etc.	Fear of darkness, of torture, terrifying black things
	Climatic Preference	Intolerance to hot and humid conditions, Summer and rainy weather	Intolerance to cold and conditions, moist conditions, Winter and rainy weather	Intolerance to dry summer and Spring	Intolerance to cold and dry conditions, late winter and autumn
	Rational Features	Reaction time slow, Continual drowsiness	Somnolence, laziness, tiredness, lassitude	Habitudes	Sense of anxiety, wakefulness
Abnormal Phenomenon	Nausea	Present	Absent	Present	Absent
	Vomiting	Absent	Absent	Yellow and green bile and “acrid flux”	Absent
	Headache	Sense of heaviness in back of eyes and temples	↑↑Sense of heaviness in back of eyes and temples	↓↓Sense of heaviness in back of eyes and temples	No sense of heaviness in back of eyes and temples
	Others	Blood flows out readily from nose, arms and gums	Weak digestion, flatulence. Cold extremities, numbness and weakness of limbs	Heart burning, Burning hands and feet	Gastric ulcers, skin cracks

Increase (↑↑) and decrease (↓↓) sign and symptoms.

3. MATERIALS AND METHOD

Study Design and Setting

This was a uni-centre, observational and prospective study conducted at Faculty of Eastern Medicine, Hamdard University, Karachi, Pakistan.

3.1. *Inclusion Criteria*

Volunteers between 19-26 years of age having no history of somatic and psychic abnormalities, with no medication consumption in past one month and native of Karachi were selected.

3.2. *Exclusion Criteria*

Volunteers below 18 year and above 30 year of age having any congenital or acquired configurational disorder and the individuals having disorders related to cardio vascular system, respiratory system, genito-urinary system, digestive system, hematology and endocrine system were excluded.

3.3. *Methodology*

Subjects (n=30) were interviewed and evaluated by random sampling method and included both genders: males (n=13) and females (n=17). The questionnaire designed for selection of volunteers included complete clinical history, general physical and systemic examination, body mass index (BMI) temperamental assessment and signs and symptoms of intermediate state.

The evaluation of temperament according to four *Akhlat* (cardinal humours) i.e *Dam* (Blood), *Balgham* (phlegm), *Safra* (yellow bile) and *Sauda* (black bile or atrabile) was carried out on the basis of set parameters which are presented in clinical trial proforma. The parameters such as body frame, touch and skin texture, complexion of the body, hairs, physical activities, dietetics, climatic preferences, excretions, sleep pattern and emotional traits, personality traits and mental activity were determined in four different types of Unani temperaments such as sanguineous *(Damvi)*, phlegmatic (*Balghami*), bilious/choleric (*Safravi*) and atrabilious/melancholic (*Saudavi*). Each parameter was qualitatively analyzed by an arbitrary score ranging from 1-10. Determination of specific temperament among four different types of temperaments can be designated by the higher score secured in each case representing as dominant temperament of the body (Zaidi, 1999).

Biochemical analyses of complete blood count (CBC) and erythrocytes sedimentation rate (ESR), lipid profile, liver function test (LFT) and fasting blood glucose (FBS) were performed after 14 hours fasting. Temperature (°F), pulse (beats/min), respiratory rate (per minute) and blood pressure (mm Hg), were measured following standard procedures. The frequencies, mean and standard error of mean of all were analyzed.

Intermediate state was measured by a questionnaire based on the general history taking and consisting of 25 questions of signs and symptoms on the basis of body temperament according to dominant body humour (*Alaamat-e-Ghalba-e-Akhlat*) which were evaluated by 6 stages ranging from health to disease (*Madarij-e-Sehat wa Marz*). Each subject was asked to select a specific statement on a six-points scale, based on how often they suffered various specific complaints in the preceding one month that were: Never, Rarely, Occasionally, Often, Very often, and Always corresponding with the continuum between health and disease: Radiant health, Not absolute health, Neither health nor disease, Potential illness, Slight ill health and Declared disease respectively.

Body weight and height were measured twice during the interview. Weight was measured in light indoor clothing without shoes on electronic scales placed on a firm leveled surface to the nearest 0.1 kg. Height was measured without shoes with a wall-mounted stadiometer to the nearest 0.1 cm. BMI was calculated as body weight (kg) divided by height (cm^2).

Data regarding socio-demographic information and health-related personality behaviors and choices were also collected during interviews. These variables were used as confounders to control for potential confounding. Demographic variables included: Age, education, occupation and marital status along with the health-related behaviors like Smoking, use of paan, gutka, supari and other physical activity.

4. RESULTS AND DISCUSSION

As per WHO statistical data only 5% of world population is enjoying healthy state of the body, 20% suffering from various diseases in which annual mortality rate is 17.5 million people for cardiovascular diseases that are estimated to be 31% of all deaths worldwide, majority of the population avoid health preservation despite experiencing tell-tale signals from their body about discomfort. Not heeding these signals can lead to more serious consequences later. The statistical data showed that the population living in between health and disease state is approximately 75% of world population, Out of which 59% of people live in dilemma about their health condition particularly the "Intermediate State" of the body. They even don't know that they are in this state of the body. 11% of the population expected to be in "Intermediate State" because of their age as understood by Jalinoos (Galen), 6% of the population have congenital defects or anomalies, 8% of the population is included in "Intermediate State" resulting defects from trauma/accidents and 16% reflected health problems by self-medication or gain advise from print or electronic media e.g: TV shows, health tips in magazines, internet etc. (Yan *et al.,* 2009).

According to Galenic medicine health is also defined in terms of mixture, mediates and balance. In the Tegni, Jalinoos (Galen) described health as the accurate amalgamation of tissues and of distinct symmetry of the body parts; explicit measures of qualities, and the precise composition in accordance to size, quality and form. It is the physician's obligation to maintain balance, restore it from unsteadiness and help to bring imbalanced bodies back to health. Jalinoos (Galen), the pioneer who described the intermediate state of the body as neutral body synonyms to *Neutrum,* as applied to bodies that can be understood in three categories: a) real intermediary between ideal, b) optimum health and c) full blown disease (Von Staden, 1989). Sub-health condition is defined by the WHO as "State between health and disease when all necessary physical and chemical indexes are negative using various diagnostic tools, apparently, however, the person experiences discomfort and even pain" (World Health Organization, 2006).

A cohort study was conducted in china on sub health status (SHS) defining it as "A state characterized by some disturbances in psychological behaviors or physical characteristics, or in some indices of medical examination, with no typical pathologic features" (Guolin *et al.,* 2013).

The objective of the study is to merge old idea of *Neutrum* with the new idea of intermediate state denoting as "I State" thus development of new preventive health procedures including innovative methods of screening counseling and treatment used as modern epidemiology, statistics, genetics and

biotechnology. The aim of the study was to identify "I State" as a distinctive trait of modernity in medicine, so that it can allows the physician to respond better to the specific needs of every patient. In this connection, it's necessary to define "I State" on the philosophical bases of Unani medicine as "A state characterized by some noticeable disturbances in physio-psychological behaviors or physical characteristics or in some parameters of biochemical fluids with no optical pathological feature."

This is a first definition of "I State" manifest Unani medicine's concept of health and disease as physio-psychological behaviors demonstrate "Temperament (*Mizaj*)" as well as physical characteristics delineates "Configuration (*Tarkeeb*)" while biochemical fluids describe "Humours (*Akhlat*)". A person is considered healthy if his *Mizaj, Tarkeeb* and *Akhlat* are in state of balance, whenever the balance in any one of these parameters is disturb the person will be considered either in state of disease or in "I State".

In this study 13 males (43.3%) and 17 females (56.7%) were evaluated by the various parameters such as; calculation of body mass index, age group distribution, analysis of temperament, analysis of temperament according to BMI, evaluation of signs and symptoms of intermediate state, physical examination and Laboratory examination.

4.1. *Body Mass Index*

The body mass index of all the Volunteers was calculated and grouped as: Normal (33.3%), low (33.3%), high (16.7%) and obese (16.7%).

4.2. *Age Groups*

All the Volunteers were distributed into two age group intervals 19-22 years and 23-26 year and further classified according to their respective BMI as shown in Fig. 1.

4.3. *The Analysis of Temperament*

The frequencies of occurrence of each temperament were obtained the results exhibited sanguineous (40.0%), phlegmatic (23.0%), choleric (20.0%) and melancholic (16.0%) respectively.

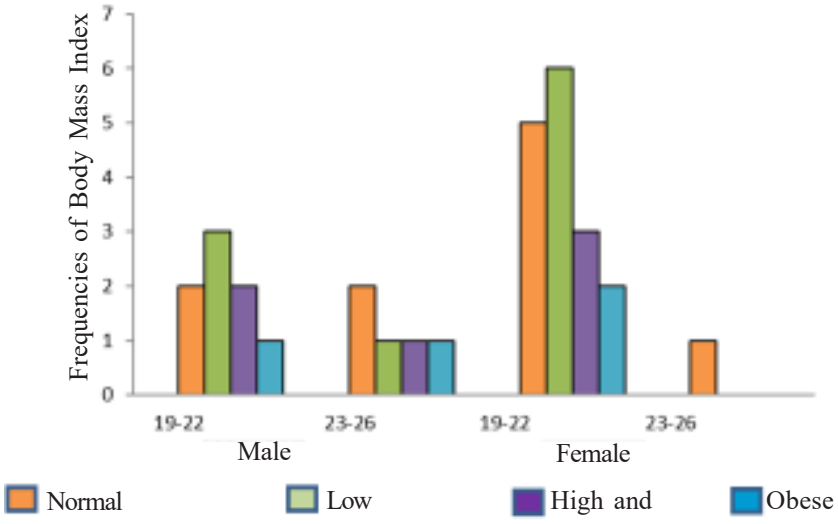

Fig. 1: Age Groups and Gender Distribution according to Body Mass Index

The higher frequency of *mizaj e damvi* is because of two reasons; one is the genetic makeup which gives them inherent dominant temperament and the second reason is age. As almost all the volunteers were belong to youth period of life (*San-e-Shabab*) which temperament is also hot and moist (*haar ratab*) so that these qualities are dominant in this period of life in individuals with other temperaments also.

The second dominating temperament is phlegmatic (*Balghami*). The explanation regarding the phlegm as second dominant humour is that all volunteers live in Karachi which is very humid city being close to the sea and meteorological data indicates that humidity is always on higher side in Karachi with the rest of country. It is understood that humidity always increases the phlegmatic secretions or (*Ratubaat-e-Badan*) in the human body. In a research study showed the dominant body temperament as sanguineous 34%, phlegmatic 40%, choleric 14% and melancholic 12%. All Volunteers who belongs to Karachi city and mostly diagnosed with the phlegmatic disorders such as respiratory system diseases. Thus, due to moisture and humidity the microbial flora prevalence is also quite higher, due to which micro-organisms can cause pathogenesis in subjects with phlegm humor as dominant body fluid more easily and actively is being increased. This all creates diseases pertaining to respiratory ailments (Syma and Hannan, 2011).

The reason for choleric temperament is also the period of life in which hot quality is being dominated. This is also justifying the least proportion of melancholic temperament which has cold and dry qualities.

4.4. *Analysis of Dominant Temperament According to BMI*

The frequencies of occurrence of dominant body temperament according to BMI were also obtained. The data shown in the Table 2 is high frequency of sanguineous (*Damvi*) temperament in normal BMI category, as well as high

Table 2: Frequency of Dominant Temperament of Volunteers According To BMI

Body Mass Index	Dominant temperament of volunteers				Total (n)
	Sanguineous	*Phlegmatic*	*Choleric*	*Atrabilious*	
Normal	6	3	0	1	10
Low	2	2	4	2	10
High	3	1	1	0	5
Obese	1	1	1	2	5
Total	12	7	6	5	30

frequency of choleric (*Safravi*) temperament in low BMI category and the melancholic (Saudavi) temperament with equal frequencies of low and obese categories of BMI. This data justify the ancient concept of human body build (*Laham wa Shaham*) that is; in sanguineous temperament there is large frame with more muscles, in phlegmatic temperament there is large frame with more fats, in choleric temperament there is medium frame with less fats and muscles (lean) and in melancholic temperament there is either thin/bony frame (tall) or large frame with strong muscles (*Tasallub e Uzlaat*) but short stature (Hussain, 1980).

4.5. *Evaluation of Signs and Symptoms*

Evaluation of signs and symptoms of intermediate state of the body were carried out on the basis of humoral/temperamental predisposition of diseases and the core concept to established signs and symptoms was the signs and symptoms of dominant humour (*Alamaat-e- Ghalba-e-Akhlat*).

The continuum between health and disease (*Madarij-e-Sehat wa Marz*) was set according to occurrence of signs/symptoms in protocol proforma, as "Never" denotes 'radiant health', "Rarely" by 'not absolute health' "Occasionally" designated for 'neither health nor disease', "Often" represents 'potential illness' whereas "Very often" indicates 'slight ill health' whilst " Always " imply 'declared disease'. The data revealed that only 3.3% found in state of radiant health while 6.7% have declared disease and a large percentage approximately 90% were found in intermediate state as experiencing different health problem so that live in "I State". Shown in Table 3.

After Galen's description of intermediate states of the body, Avicenna best described the stages ranging from health to disease, as radiant health, not absolute health, neither health nor disease, potential illness, slight ill health, and declared disease (Gruner OC 1973). Through this study first time in the world we can also set a criterion for clinical diagnosis of "I State" on the basis of some signs and symptoms specify imbalance in humors/temperament such as fatigue, headache, dizziness, eye strained heart burning, poor appetite, indigestion, running nose, sore throat, shortness of breath, hair fall, anxiety, depression, musculoskeletal pain, constipation, lack of concentration, short memory, sleeplessness, excessive dreaming, thermal intolerance, palpitation, profuse sweating, on and off diarrhea and constipation.

The clinical picture of "I State" can be correlated with signs and symptoms of dominant humor or predisposition of diseases of dominant temperament, such as this study showed the symptoms e.g.: easily fatigue or lethargic, poor stamina, easily annoyed or perplexity constipation are common in phlegmatic temperament whereas heart burning, burning hands and feets, aggressiveness are commonly associated with bilious temperament, as well as the symptoms like, palpitation, facial pain, profuse sweating are found in sanguineous temperament while irritability, insomnia, low concentration, depression are generally associated with melancholic temperament. The etiology of "I State" may be affected by lifestyle governing factors (*Asbab-e-Ditta Zaroriya*) and re-modification of the same may prevent "I State." In this regard, "I state" seems to be more multifaceted complex condition.

4.6. *Physical Examination*

The temperature, pulse, respiratory rate (TPR) was recorded for all the volunteers. In order to validate the differences amongst the categories of body mass index (BMI) the Mean±SEM of all physical examination

Table 3: Frequencies and Percentages of Signs and Symptoms of Intermediate State of the Body

S.No.	Signs and Symptoms	Continuum between Health and Disease (*Madarij-e-Sehat wa Marz*)					
		Radiant health (Never)	Not absolute health (Rarely)	Neither health nor disease (Occasionally)	Potential illness (Often)	Slight ill health (Very often)	Declared disease (Always)
1.	physical activity and stamina	4 (13.3)	8 (26.7)	12 (40)	5 16.7)	1 (3.3)	0 (0.0)
2.	Easily fatigue/ lethargic	1 (3.3)	9 (30)	10 (33.3)	9 (30)	1 (3.3)	0 (0.0)
3.	Hair fall	3 (10)	12 (40)	10 (33.3)	1 (3.3)	3 (10.0)	1 (3.3)
4.	Headaches	0 (0.0)	10 (33.3)	14 (46.7)	2 (6.7)	1 (3.3)	3 (10.0)
5.	Acne/Pimples	20 (66.7)	10 (33.3)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
6.	Eyes ache or feel tired/ Swollen eyelids	2 (6.7)	8 (26.7)	9 (30.0)	10 (33.3)	0 (0.0)	1 (3.3)
7.	Sore throat	4 (13.3)	8 (26.7)	9 (30.0)	3 (10.0)	0 (0.0)	6 (20.0)
8.	Muscles or joints feel stiff	10 (33.3)	1 (3.3)	5 (16.7)	13 (43.3)	1 (3.3)	0 (0.0)
9.	Pain in shoulders/ neck/waist	14 (46.7)	1 (3.3)	4 (13.3)	6 (20.0)	2 (6.7)	3 (10.0)
10.	Heavy feeling in your legs when walking	13 (43.3)	7 (23.3)	4 (13.3)	3 (10.0)	0 (0.0)	3 (10.0)
11.	Feel out of breath while resting	15 (50.0)	5 (16.7)	5 (16.7)	3 (10.0)	0 (0.0)	2 (6.7)
12.	Chest congestion/cough	2 (6.7)	6 (20.0)	2 (6.7)	10 (33.3)	8 (26.7)	2 (6.7)
13.	Heart palpitations	7 (23.3)	10 (33.3)	6 (20.0)	3 (10.0)	3 (10.0)	1 (3.3)

S.No.	Signs and Symptoms	Radiant health (Never)	Not absolute health (Rarely)	Neither health nor disease (Occasionally)	Potential illness (Often)	Slight ill health (Very often)	Declared disease (Always)
14.	Change in appetite/ thirst	5 (16.7)	1 (3.3)	8 (26.7)	4 (13.3)	11 (36.7)	1 (3.3)
15.	Heart burning	1 (3.3)	9 (30.0)	3 (10.0)	6 (20.0)	11 (36.7)	0 (0.0)
16.	Nausea/vomiting	1 (3.3)	8 (26.7)	5 (16.7)	4 (13.3)	9 (30.0)	3 (10.0)
17.	Difficulty tolerating the cold/heat	10 (33.3)	10 (33.3)	2 (6.7)	3 (10.0)	3 (10.0)	2 (6.7)
18.	Insomnia/somnolence	15 (50.0)	9 (30.0)	4 (13.3)	2 (6.7)	0 (0.0)	0 (0.0)
19.	Upset GIT/ constipation/diarrhea	8 (26.7)	9 (30.0)	7 (23.3)	4 (13.3)	2 (6.7)	0 (0.0)
20.	Trouble with short-term memory	1 (3.3)	6 (20.0)	14 (46.7)	3 (10.0)	5 (16.7)	1 (3.3)
21.	Difficulty responding quickly	0 (0.0)	14 (46.7)	6 (20.0)	8 (26.7)	2 (6.7)	0 (0.0)
22.	Difficulty in concentration	0 (0.0)	12 (40.0)	8 (26.7)	5 (16.7)	5 (16.7)	0 (0.0)
23.	Numbness of limbs/ weak limbs	0 (0.0)	12 (40.0)	10 (33.3)	5 (16.7)	3 (10.0)	0 (0.0)
24.	Nervousness	1 (3.3)	10 (33.3)	11 (36.7)	7 (23.3)	1 (3.3)	0 (0.0)
25.	Cold/burning extremities	3 (10.0)	15 (50.0)	4 (13.3)	4 (13.3)	4 (13.3)	0 (0.0)

Values within parenthesis are percentages.

n = 30

As the epidemiological statistics of WHO and study has been conducted in China showed that there are some remarkable signs and symptoms which indicate that person is neither in state of radiant health nor diseased but he/ she living in between these states (WHO, 2006).

Table 4: Body Temperature, Pulse Rate, Systolic Blood Pressure, Diastolic Blood Pressure According to Body Mass Index

Body Mass Index (BMI)	Body temperature (°F)	Systolic blood pressure (mmHg)	Diastolic blood pressure (mmHg)	Pulse rate	Respiratory rate
Normal	95.18±2.15	112.40±17.97	77.80±18.48	86.40±12.52	12.60±0.86
Low	96.44±1.85	110.60±14.86	72.40±8.66	92.10±9.56	12.04±0.78
High	96.44±3.21	121.20±22.97	75.20±7.46	101.80±11.73	12.56±0.86
Obese	97.16±1.97	115.00±14.89	73.60±12.46	98.20±12.11	11.96±0.20

Values are Mean±S.E.M. of n = 30.

Percentage: Normal and Low (33.3%) while High and Obese (16.7%).

BMI: Normal (18.5- 24.9), Low (less than 18.5), High (25-29.9) and obese (30 or more).

Body Temperature (°F): The average normal body temperature is 98.6°F (37°C).

Systolic Blood Pressure (mmHg): Normal blood pressure: below 120 mmHg.

Diastolic Blood Pressure (mmHg): Normal blood pressure: below 80 mmHg.

Pulse Rate: 72- 80 beats/ minutes.

Respiratory Rate: The normal respiration rate for an adult at rest is 12 to 20 breaths per minute.

parameters are shown in Table 4, exhibited that the descriptive values of temperature, pulse rate, respiratory rate and systolic and diastolic blood pressure were within normal ranges

4.7. *Laboratory investigations*

Biochemical analyses of complete blood count (CBC) and erythrocytes Sedimentation Rate (ESR), lipid profile, liver function test and fasting blood glucose were performed after 14 hours fasting.

The CBC was carried out and the results of all volunteers were analyzed and Mean ± SEM was obtained. The data was then evaluated on the basis of four categories of body mass index as normal, low, high and obese respectively. The data shown in Table 5 no significant difference among the four categories of BMI but laboratory reports shown some morphological variations in blood cells, on the basis of which remarks and advises were given by pathologist.

Table 5: Complete Blood Count According to Body Mass Index

Lab. tests	Parameters	Body Mass Index			
		Normal	Low	High	Obese
Complete Blood Count	Hemoglobin (mg/dl)	12.36±0.92	12.94±1.69	13.16±2.05	13.60±2.28
	Red Blood cells (10^6/µl)	4.83±0.81	4.88±0.44	4.76±0.74	4.73±0.53
	Hematocrit (%)	39.27±3.21	39.91±4.46	41.92±5.09	41.38±5.99
	Mean corpuscular Volume (fl)	82.53±9.39	81.86±6.27	86.78±3.84	87.32±3.73
	Mean Corpuscular Hemoglobin (pg)	26.00±3.23	26.53±2.62	27.14±1.25	28.66±2.71
	Mean Corpuscular Hemoglobin Concentration (g/dl)	31.50±0.82	32.35±1.44	31.30±1.32	32.72±1.73
	Total Leucocyte Count (10^3/ul)	7.83±2.40	7.04±2.36	7.92±0.96	8.42±1.22
	Neutrophils (%)	58.30±8.66	54.90±3.75	56.40±5.41	55.60±3.04
	Lymphocytes (%)	34.20±7.26	37.00±3.16	37.60±4.77	37.40±3.43
	Eosinophils (%)	2.50±2.67	2.40±0.84	2.40±1.67	2.20±1.09
	Monocytes (%)	5.00±1.49	5.70±1.15	3.60±2.07	4.80±1.92
	Basophils (%)	.000±.000	.000±.000	.000±.000	.000±.000
	Platelets Count (10^3/ul)	243.90±54.55	260.00±50.59	270.20±46.09	299.40±74.93
ESR	Erythrocytes Sedimentation Rate (mm/1st hr)	*29.70±18.30	*20.40±11.02	*25.40±12.38	*19.20±6.26
FBS	Fasting Blood Sugar (mg/dl)	91.10±7.43	86.80±5.26	91.80±5.67	94.20±5.26

Values are Mean±S.E.M. of n = 30.

Percentage: Normal and Low (33.3%) while High and Obese (16.7%).

BMI: Normal (1.5-24.9), Low (<18.5), Higher (25-29.9) and Obese (30 or more).

Complete Blood Count: Erythrocytes (Normal ranges): Hemoglobin (mg/dl): 11.5-16.4, RBCs (10^6/ul): 3.9-5.6, Hematocrit (%): 36-46, MCV(fl): 76-96, MCH (pg): 28-32, MCHC (g/dl): 32-36. Leucocytes (Normal Ranges): Total Leucocyte count (10^3/µl): 4.0-11.0, Neutrophils (%): 40-75, Lymphocytes (%): 20-45, Eosinophil (%): 1-6, Monocytes (%): 2-10, Basophils (%): 0-1. Thrombocyte (10^3/µl) (Normal range): 150-400,

Erythrocytes Sedimentation Rate (ESR) (mm/1st hr) (Normal range): Male 0-10, Female 0-18.

*Moderately elevated ESR occurs with inflammation but also with anemia, infection, pregnancy, and with aging.

Fasting Blood Sugar (FBS) (mg/dl): (Normal range): 70-110.

The erythrocytes sedimentation rate (ESR) of all volunteers was also done and the Mean±SEM were then obtained. The Table 5 showed the mean values of ESR was even highest in the normal body mass index category which was clearly indicated any concealed pathological condition in the body. This data also confirmed the evaluation of signs and symptoms on the basis of dominant temperament, through which volunteers exhibited their discomfiture and even pain in their daily life without explaining any diagnosed disease condition. Though, data revealed persons seems normal but actually they were not.

The fasting blood sugar (FBS) was also done and the results were analyzed by Mean±SEM. The data showed the highest values obtained in obese person according to BMI that is 94.20±5.26 while decreased in low BMI as 86.80±5.26. As shown in Table 5. It is concluded that with in normal range the values of blood glucose level are varies. As glucose is a major source of providing energy in the body and lack of glucose in the blood will affect the physical activity of the body. It also exhibited the diagnostic sign of the dominant body temperament as sanguineous are moderately active and has good stamina and physical endurance. Phlegmatic are very prone to lethargic easily, slow in action and has poor stamina. As well as choleric are very active, enthusiastic and has very good stamina (Kabeeruddin, 1930). As the results showed in this study the frequency of occurrence of choleric temperament is increased in low category of BMI. Though, it concluded that as the people with choleric temperaments are more active so that utilization of energy or glucose consumption is greater in this temperament that may be a reason for less glucose level in the blood. But it also exhibit that the need of glucose is more in this temperament as well.

As shown in Table 6 the liver function test and lipid profile was carried out and the results of all volunteers were analyzed and Mean±SEM of all biochemical parameters were obtained. The data was then evaluated on the basis of four categories of body mass index. There is no significant differences among four categories of BMI in all parameters are shown in Table 6 but comparatively some higher values observed the high and obese categories especially of lipid profile parameters. It exhibited that these volunteers were living in any stage of intermediate state.

In both genders, there was a positive correlation between dominant body temperament and body mass index (BMI) with laboratory investigations remarks and advises. The laboratory data exhibited in males 1 volunteer advised Hb Electrophoresis, Thalassemia Minor and remarks was Fatty Liver disease, 1 advised for Stool D/R and IgE level with remarks of Eosinophilia. As well as in case of females 1 advised for Hb Electrophoresis to scrutinized Thalassemia Minor, 1 for viral marker, 1 diagnosed with Leucocytosis as well as 1 with Reactive Neutrophilic Leucocytosis whereas 11 males and 14 females were found with raised ESR levels suggested further clinical evaluation. Shown in Table 7.

Although epidemiological studies and statistics showed some data about the “I State” but WHO does not recommended any lab investigation for the diagnosis of “I State” though for better understanding considere this; diabetes test is positive if glucose concentration is greater than 140 mg/dl in blood after over night fast. Normal range of glucose in the blood is 70 to 100 mg/dl. Would a person be normal if result is 101 to 139 mg/dl? With this simple justification through this study the existence of “I State” can be confirmed by performing general physical examination, temperature, pulse, respiratory rate

Table 6: Liver Functions Test and Lipid Profile According to Body Mass Index

Lab. tests	Parameters	Body Mass Index			
		Normal	Low	High	Obese
Liver Functions Test	Total Bilirubin (mg/dl)	0.89±0.58	0.65±0.35	0.50±0.10	0.80±0.38
	Direct Bilirubin (mg/dl)	0.265±0.17	0.22±0.09	0.19±0.02	0.25±0.10
	Indirect Bilirubin (mg/dl)	0.62±0.42	0.43±0.26	0.31±0.07	0.55±0.29
	Serum Glutamic-pyruvic transaminase (U/L)	16.10±6.52	15.70±7.46	33.00±21.13	31.00±27.08
	Alkaline Phosphatase (U/L)	74.90±23.41	66.30±13.60	85.20±13.60	88.20±33.46
Lipid Profile	Gamma GT (U/L)	13.70±5.10	13.40±3.20	20.40±8.08	24.60±16.14
	Total Cholesterol (mg/dl)	152.20±22.82	145.10±22.28	172.00±31.16	179.00±26.55
	Triglycerides (mg/dl)	98.30±66.70	79.80±27.16	111.20±32.42	146.40±105.97
	High Density Lipoprotein (mg/dl)	57.20±23.83	48.50±13.20	47.60±7.43	41.80±7.85
	Low Density Lipoprotein (mg/dl)	81.00±29.39	84.50±16.91	109.40±29.03	114.20±27.05

Values are Mean±S.E.M. of n = 30.

Percentage: Normal and Low (33.3%) while High and Obese (16.7%).

Liver Function Test (Normal ranges): Total bilirubin (mg/dl): <1, Direct bilirubin (mg/dl): <0.30, Indirect Bilirubin (mg/dl): <0.5, SGPT (U/L): In male upto 41, in females upto 33, Alkaline phosphatase(U/L): male 40-130, females 35-105, Gamma GT (U/L): 10-50.

Lipid Profile (Normal ranges): Cholesterol (mg/dl): <200, Triglycerides (mg/dl): <150, HDL (mg/dl): 45-65, LDL (mg/dl): <130.

and blood pressure as well as calculation of BMI and some diagnostic investigations like, complete blood count, erythrocytes sedimentation rate (ESR), liver function test, lipid profile, fasting blood glucose and also urine D/R. Similarly, all biochemical fluids have their ranges through which they exhibited very thin line of demarcation amongst different states of the body viz; health, intermediate state and disease.

The cycle of states of the body is elaborated in Fig. 2. The body has a natural propensity to restore balance in humors, but minor imbalances in humors cause discomfort,

Table 7: Remarks and Suggestions on Lab Investigations According to Body Mass Index and Gender of the Volunteers Cross Tabulation

Gender of the Volunteers	Advised/Remarks on Lab Investigations	Body Mass Index				
		Normal	Low	High	Obese	Total
Male (n=13)	Hb Ectrophoresis, Thalassemia Minor, Fatty Liver disease	1	0	0	0	1
	Stool D/R, IgE level, Eosinophilia	1	0	0	0	1
	Further Clinical Evaluation required	2	4	3	2	11
Females (n=17)	Hb Electrophoresis,					
	Thalassemia Minor	0	1	0	0	1
	Viral Marker	0	0	0	1	1
	Inc. ESR required more History	0	1	0	0	1
	Leucocytosis	0	1	0	0	1
	Reactive Neutrophilic Leucocytosis	1	0	0	0	1
	Further Clinical Evaluation required	5	3	2	2	12

Percentage: Normal and Low (33.3%) while High and Obese (16.7%).

and major imbalances result in disease. As a disease starts it shows minor symptoms. These symptoms when catch up their full capacity leads to the 'increment' in the intensity of the disease. This intensity increases till a point of constancy occurs. At this stage, if physis (*Tabiyat Mudabbara-e-Badan*) is strong enough to be able to overcome that disease, the body is able to regain its state of health. On the other way, the disease either turns in to chronic disease or the death of the patient occurs. Most people simply assume that after a disease, the state of health achieved directly but this is not the case. According to Unani system of medicine, there is an intermediate

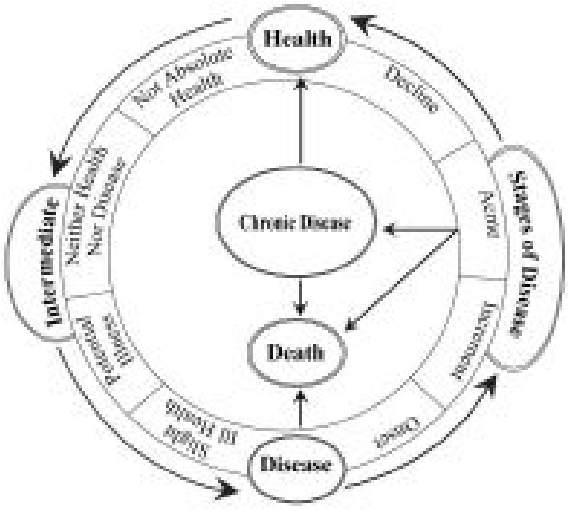

Fig. 2: Cycle of states of the body

state of the body which is designate in between the extremes of absolute health and declared disease. It is actually the state in which illness varies in severity. This state shows that death does not occur suddenly, there are some yawning and yelling in the body which was ignored.

5. CONCLUSION

This study not only exhibited the Jalinoos (Galen)'s concept of states of the body; health, disease and intermediate state but further classified intermediate state in which person is neither totally sick nor totally healthy. Diverse clinical situation lays emphasis on severity of these stages.

As Unani Medicine is based on Hippocratic theory of "Rule of harmony" indicating that health is a harmonious balance of the four humors and reflects that disease is the result of disharmony and imbalance. Thus, the present study also denies the modern medicine concept of 'sudden death' and further emphasizes more on the role of six essential factors for healthy lifestyle, maintenance and preservation of the health condition.

Conflict of Interest

The authors declare that there are no conflicts of interest.

6. ACKNOWLEGMENT

I would like to thanks Mr. Ahmad Yar Sukhera, President, Research and Exhibition Society, Faculty of Eastern Medicine, Hamdard University for sharing valuable knowledge and collecting data to make this effort possible.

Hussaini Blood Bank for their Support and Collaboration.

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Pharmaceutical Evaluation of Mefenamic Acid Tables Available in Local Market of Rawalpindi Islamabad, Pakistan

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Mefenamic acid is a commonly prescribed non-steroidal anti-inflammatory drug in mild to moderate pains. Different multinational and national brands of mefenamic acid are available and are being frequently prescribed in Rawalpindi and Islamabad, Pakistan. Pharmacies and dispensaries associated with clinics usually dispense local brands which are cost effective as compared to multinational brands. Present study was conducted to assess pharmaceutical equivalence of one multinational (X1) and four national brands namely, X2, X3, X4 and X5, so as to prove their use as an alternative to one another. All the brands were evaluated for their quality control parameters of hardness, friability, weight variation, disintegration, dissolution and assay of active ingredient. All the brands fulfilled quality control criteria's of USP while 2 of the national brands did not meet B.P standards of assay of active ingredients by brands X1, X4 and X5 were pharmaceutical equivalents and can be substituted one another. Results of the study reveal that good manufacturing practice guidelines are dominantly being followed in multinational as well as national pharmaceutical companies.

Keywords

Mefenamic acid, Assay of active ingredient, Good manufacturing practice.

1. INTRODUCTION

Mefenamic acid (MA) is a non-steroidal anti-inflammatory drug (NSAID) which is commonly used for the treatment of pain and inflammation. It is an official drug in *British Pharmacopoeia* (B.P.) and is regularly prescribed for the prophylaxis of headache and premenstrual migraine and period cramps (Pringsheim *et al.,* 2008). Mefenamic acid 2-(2,3-dimethylphenyl) amino benzoic acid is a white to yellowish microcrystalline powder having melting point 230-231°C. It belongs to the Biopharmaceutical Classification System (BCS) class II drugs having less water solubility but greater permeability. To some extent this drug is soluble in alcohol and methylene chloride (Commission, 2009; Nurhikmah, 2016).

Mefenamic acid

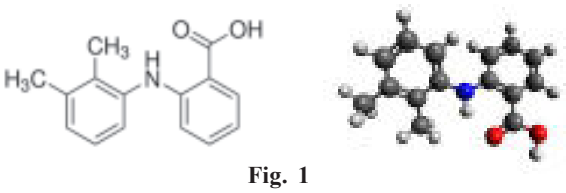

Fig. 1

Mefenamic acid acts as an analgesic and anti-inflammatory agent by competitively

inhibiting cyclo-oxygenase COX 1 and COX 2, and thereby decreasing the production of prostaglandin. Many national and multinational brands and dosage forms of mefenamic acid are available in Pakistan. Various brands of mefenamic acid available in market are considered Pharmaceutical Equivalent. Pharmaceutical equivalence is the condition in which drug products contain identical quantity of active ingredients, in an identical dosage form, meeting all applicable compendia requirements of quality, strength, potency and purity, however, they may vary in characteristics such as excipients, shape, packaging, expiry date, and labeling requirements etc (Chen *et al.,* 2001). Availability of pharmaceutical equivalents helps the pharmacists and practitioners in substitution of brand in case of non availability of any brand; whereas, such substitutions often become controversial and is met with suspicion among physicians and patients (Meredith, 2003).

Mefenamic acid is a highly marketed generic in Pakistan, and many brands are available in local market but unfortunately demonstrates different therapeutic responses and ultimately cause dissatisfaction of patient as well as the practitioner. The variation in therapeutic response is most likely due to difference in excipient's nature, concentration and variation in quality control standards of multinational and national brands. A quality medicine is required for the proper therapeutic response; otherwise a quality compromised drug not only cause financial loss but ultimately life threatening consequences (Shakoor, 1997). Various national pharmaceutical companies are producing MA tablets in very low cost as compared to the multinational brands. Present study is aimed to find pharmaceutical equivalence of such national brands of Pakistan with a multinational brand available in local market. The study will help practitioners and pharmacists in selection of most cost effective and therapeutically active brand.

2. MATERIALS AND METHODS

One multinational and four national brands of MA were purchased from three different community pharmacies of Islamabad and Rawalpindi cities of Pakistan. Analytical standard of MA was obtained from Sigma Aldrich. Multinational brand was coded as X1 while others as X2, X3, X4 and X5. *In vitro* bioequivalence was determined by performing dissolution test and assay of active ingredient along with hardness, friability, weight variation and disintegration tests.

Table 1: Brands of Mefenamic Acid Used

Code	Brand	Batch Number	Registration Number
X1	Ponstan	1652613	000138
X2	Mefnac	K047	004481
X3	Kamic	K387	043343
X4	Namcid	646	034640
X5	Ponsid	379	060772

Strength in all brands = 250 mg

2.1. *Friability Test*

Randomly selected 10 tablets from each brand were initially weighed and introduced into Roche friabilator. The apparatus was operated on 25 rpm for 4 mins. After completion of cycle tablets were dedusted and finally weighed. Final weights were compared with initial weights of

the tablets and friability was determined using following formula (Pharmacopoeia, 1998):

$$\text{Friability \%} = \frac{\text{weight before test} - \text{weight after test}}{\text{weight before test}} \times 100$$

2.2 *Weight Variation*

Randomly selected tablets (n=20) from each brand were weighed individually by using Sartorious digital weighing balance and calculated the average weight. Then determined the percentage variation from average weight by using following formula (Pharmacopoeia, 2007).

$$\text{Weight Variation \%} = \frac{\text{Individual weight} - \text{Average weight}}{\text{Average weight}} \times 100$$

2.3 *Hardness Test*

Tablet strength of 10 randomly selected tablets of each brand was measured using Monsanto hardness tester. Each tablet was pressed between the two anvils until the tablet broke into pieces. Value of applied force at breaking point was noted and mean was calculated (Albreiki, 2013).

2.4. *Tablet Disintegration Test*

Disintegration test was performed on randomly selected 6 tablets of each brand. One tablet was placed in each tube of the basket and disc was placed to avoid upward and downward movement of the tablet. Apparatus was operated at 37±2°C using distilled water as medium. Disintegration time of each brand of tablet was noted at point where no part of the tablet remained on the sieve (Albreiki, 2013).

2.5. *Dissolution Test*

Dissolution testing on randomly selected 6 tablets of each brand was performed in a rotating basket type USP dissolution test apparatus. One tablet was added in each of six vessels containing 900 ml of distilled water as dissolution media. Basket was rotated at 50 rpm at 37±0.5°C for 45 min. Aliquots (3.0 ml) was withdrawn after 45 min., filtered and diluted with NaOH (0.1 M). Percentage of drug dissolved was determined using UV/Visible spectrophotometer (JASCO V730) (n=3). The amount of drug dissolved was computed against 5 µg/ml of standard (Absorbance=0.2543) (Albreiki, 2013).

2.6. *Assay of active ingredient*

Tablets (20) were selected randomly, weighed individually and calculated their average weights crushed with the help of pestle and mortar until a fine powder was obtained. Powder equivalent to 500 mg of mefenamic acid was taken and dissolved in 60-70 ml of ethanol. Sodium hydroxide (250 ml of 0.1 N) was prepared and filled in the burette and then standardized against oxalic acid (0.1 N) before titration with mefenamic acid which was taken in titration flask and phenol red indicator was added in it. Gradually NaOH solution was introduced from burette to mefenamic Acid solution until end point is appeared which showed the consumption of Mefenemic acid. Each ml of sodium hydroxide (0.1 M) is equal to 24.13 mg of mefenamic acid (Commission, 2009). Then same procedure was repeated 3 times for each brand and their percentage purity was calculated by following formula:

$$\text{Percentage purity} = \frac{\text{Calculated Weight}}{\text{Actual Weight}} \times 100$$

2.7. *Data Analysis*

Data was analyzed by calculating mean± standard deviation for all tests (Friablity, weight variation test, hardness test, dissolution time, disintegration time and assay of active ingredient).

3. RESULTS AND DISCUSSION

The results of different test are presented in Table 2.

Table 2: Quality Control Parameters for Selected Brands of Mefenamic Acid

Brand Codes	Friabilty (%)	Hardness (kg/cm^2)	Weight variation (g)	Disintegration time (Minute)	Drug dissolved (%)	Assay of active ingredient (%)
X1	0.76±0.04	5.38±1.05	0.522±0.004	0:32	95.5±3.50	98.5±2.42
X2	0.10±0.01	7.03±1.65	0.360±0.054	2:33	86±2.90	92.2±3.76
X3	0.76±0.05	8.55±1.43	0.467±0.029	6:23	88.6±4.86	93.5±4.50
X4	0.91±0.06	5.9±0.85	0.570±0.068	1:17	92.9±6.73	95.2±6.62
X5	0.23±0.03	8.0±1.70	0.595±0.041	1:05	88.6±2.46	96.4±2.44
n	10	10	20	6	6	3

Percentage friability of all brands were within range of 0.10±0.01 to 0.91±0.06. None of the brand crossed USP limit of 1%. Hardness of all brands were in the acceptable range of 5.38±1.05 to 8.55±1.43. One tablet each from X3 and X5 crossed the upper specified limit of 10 kg/cm^2 (Allen and Ansel, 2004). The result of weight variation test was within acceptable limit while is only one tablet each from X2, X4 and X5 deviated from the USP limit of ±5%. All formulations disintegrated very rapidly except X3 which took 6 mins and 23 seconds. Still all values were well within standard limits. The disintegration time ranged from 32 sec to 6:17 mins which was within B.P limit of 15 mins for uncoated tablets (Pharmacopoeia, 2013). Result of dissolution test was found well within USP and BP limits (Anderson *et al.,* 1998). Percent drug dissolved for all brands were between 86±2.90% to 95.5±3.50%. Assay of active ingredient for all brands were also within range (92.2±3.76%-98.5±2.42%), which we acceptible by the quality control standard criteria of USP while X2 and X3 did not meet B.P. specifications of assay. According to BP the product should lie within 95%-105% of active content.

All the brands were within the acceptable limit of quality control standards prescribed by USP and B.P except that X2 and X3 did not meet B.P standards for assay of active ingredient. Multinational brand was found most closer to the claimed limits and fulfilled quality control standards with minimum variation. Similar study was performed on 6 brands of 250 mg mefenamic acid tablets obtained from Karachi, Pakistan where 5 local brands were compared with a multinational brand. All the local brands fulfilled the standard criteria of quality control parameters (Zafar *et al.,* 2015).

Results are consistent with our findings in all parameter except assay of active ingredient. This variation may be due to use of active ingredient which was not meeting B.P standards or the inappropriate humidity and temperature control during storage and production process which degraded the product. Another study was performed in Oman where 5 brands of mefenemic acid were assessed for their pharmaceutical equivalence. One local brand did not meet B.P. standard of assay of active ingredient and was not considered pharmaceutical equivalent of other brands. All other brands were gave satisfactory results (Albreiki, 2013). This result is similar to our findings. Studies performed on brand comparison of different brands of ciprofloxacin, domperidone, ranitidine, paracetamol and levofloxacin available in Pakistan also showed similar results (Azeem *et al.,* 2015; Khan *et al.,* 2014; Naveed *et al.,* 2014; Londhe and Desai, 2013; Bashir *et al.,* 2015). Few locally manufactured brands did not fulfilled quality control parameters. Multinational brands met quality control standards because they are well versed with facilities and resources to meet the GMP's. National drug manufacturers financial resources to purchase best purity grade raw materials, and hire required man power to fulfill needs of the regulatory bodies. Frequent change in staff and personal shortcomings in expertise of personnel is also a contributing factor following GMP compliance. Any shortcoming in meeting quality control standards is due to lack in adherence to good manufacturing practice and not due to fraudulent intention (Shakoor, 1997).

4. CONCLUSION

It is concluded that all national brands are fulfilling USP and B.P requirements except that X2 and X3 failed to meet B.P standards of assay of active ingredient. Brands X1, X4 and X5 are considered pharmaceutical equivalents and can be prescribed alternatively. Patients can switch from one brand to another in case of any non compliance issue.

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Scientific Findings of Pakistan Origin Halophyte *Launeae nudicaulis* (L.) Hook.f.

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Abstract

In arid and semi-arid regions of the world salinity is the one of the major problem that causes tremendous loss in the crop yield. It is required to bring these areas under utilization for maximizing the crop production either by removing salinity or by using salt tolerant crops. These plants are growing on the soil as creepers and herbs such as *Citrullus colocynthis*, *Cres erotica*, *Launeae nudicaulis* (L.) Hook.f. etc. One of such kind of plants, *L. nudicaulis* (L.) Hook.f. has been selected for the current research study. *L. nudicaulis* (L.) Hook.f. is abundantly found in Pakistan, especially in Karachi, parts of Sindh province and Cholistan desert. The *L. nudicaulis* (L.) Hook.f. Compositae (Asteraceae) is considered as facultive type of Halophyte which can grow on saline as well as non saline soil. According to the literature survey, this creeper facultive halophyte has great medicinal significance. It is recommended in Folk medicine as bitter tonic, stomachic, wound healing accelerator etc. Furthermore, it is reported to possess antioxidant, antimicrobial, insecticidal and cytotoxic properties. Present study is based on standardization, phytochemical, urease inhibition and heavy metal analysis of three morphological parts of *L. nudicaulis* (L.) Hook.f. i.e. (A) root, (B) stems, and (C) leaves. Due to significant medicinal properties, agronomy and easy accessibility of this ignored halophyte, preliminary phytochemical, physico-chemical, moisture content, total ash content and florescent analysis of all parts of plant were separately performed to establish the standard protocol for its identification that is crucial for medical use and determination of existing chemical components either primary or secondary metabolites in each part and their behaviors. Elemental analysis and urease inhibition activity were carried out to prove this plant as both nutritional and agronomic potential along with their toxic components present in each part of *L. nudicaulis* (L.) Hook.f.

Keywords

Launeae nudicaulis (L.) Hook.f., Halophyte, Urease inhibition, Fluorescence analysis, Physico-chemical analysis.

1. INTRODUCTION

Pakistan is about 80,943 km^2, located between 23° 45' to 36° 50' N latitude and 60° 55' to

75° 30' E longitude and has altitude ranging from 0 to 8611 m. Thereby it has not only variety of climate zones but also unique biodiversity. About 6000 higher plant species are present from which 600 to 700 are used for medicinal purposes (Ali and Qaiser, 1986). Pakistan has centuries old knowledge about traditional uses of plants for different kinds of diseases which has been transferred from generation to generation. The traditional knowledge about plants and their medicinal uses also reflects the values embedded in traditions that is upheld and transferred by elders. The major reason of use of these plants in treatment and cure of diseases is that they contain negligible side effects or synergistic effects neutralizing combinations in them (Gilani and Atta-ur-Rahman, 2005). While there is major concern about non beneficial or harmful effects of synthetic chemicals that are being used in treatment; Hence now the trends are changing towards the use of natural origin medicinal products. Many of these natural medicinal plants are perennial halophytes including some herbaceous annuals. Halophytes are classified into 3 main catagories that is True, Facultative and Glycophyte on the basis of adaptability of saline soil. *L. nudicaulis* (L.) Hook.f. includes facultive type of halophytes as optimal growth can be attained on saline soil as well as non saline soil (Sen *et al*., 1982).

In Pakistan more than 400 species of halophytes have been reported with 100 species and almost 25% have been reported from coastal areas (Khan and Qaiser, 2006). These are of much significant economic potential and can contribute extensively towards the restoration of environment besides potential medicinal source (Muhammad *et al*., 2011). Literature review shows an increase in interest in research on halophytes which recognized it as a valuable resource and cash crop of immense potential (Qasim *et al*., 2011; Hussain *et al*., 2003). Medicinal uses of few common Halophytes are listed in Table 1.

Table 1: Medicinal Uses of Important Halophytes Naturally Growing on Salty Soils

Species	Medicinal uses
Acanthus ilicifolius (L.)	Rheumatism, neuralgia, paralysis, asthma and as blood purifier
Achyranthes aspera (L.)	Asthma, renal dropsis
Acrostichum aureum (L.)	Applied on wounds and boils
Adhatoda vasica(L.)	Asthmatic problems
Aloe barbadensis Miller	Piles, rheumatism, boils and stomach problems.
Barringtonia acutangula (L.)	Diarrhoea, toothache
B. racemesa (Lam.)	Cough, asthma, diarrhoea, jaundice
Calophyllum inophyllum (L.)	Seed oil in rheumatism, skin diseases and leprosy
Calotropis procera Ait.	Skin diseases, tumours, piles, as abortifacient and anticoagulant

Species	Medicinal uses
Capparis deciduas (Forsk.)	Cough, asthma, inflammations, cardiac troubles and biliousness
Cerbera manghas (L.)	Rheumatism
Citrullus colocynthis (L.)	Jaundice, rheumatism and urinary diseases.
Clerodendrum inerme (L.)	Malarial fever as substitute to quinine
Cress cretica (L.)	Tonic, aphordisiac, stomachic
Cynometra ramiflora (L.)	Leprosy, scabies and cutaneous diseases
Heritiera fomes buch	Piles
H. littoralis Aiton	Diarrhoea and dysentery
Jatropha curcas (L.)	Diarrhoea, toothache, piles, rheumatism and skin diseases
Kochia indica Wight	Cardiac stimulant
Pandanus odoratissimus (Frossk)	Leprosy, scabies, diseases of heart, oil as antispasmodic
Pongamia pinnata (L.)	Diarrhoea, cough, leprosy, gonorrhoea, rheumatic pains
Ricinus communis (L.)	Boils, sores, lumbago
Salsola baryosma, S. kali	Posses anthelmintic, emmenagogue, diuretic properties, ash for itch
Salvadora persica, (L.)	Cough, rheumatism, suppositories, toothache and piles
Solanum surattense Burm. f.	Cough, asthma, sore throat, rheumatism, fever
Sonneratia caseolaris (L.)	Vermifuge
Suaeda fruticosa (L.) forssk	Emetic and to cure sores on camel back
Tamarix articulata vahl	Eczema, ulcers, piles, sore throat, diarrhoea, liver disorders
Terminalia catappa (L.)	Cutaneous diseases
Thespesia populnea (L.)	Stomach trouble
Trianthema portulacastrum (L.)	Asthma, amenorrhoea, dropsy, rheumatism, liver problems, and as abortifacient
Tribulus terrestris (L.)	Tonic, diuretic and in painful micturition and calculous affections
Vetiveria zizanioides(L.)	Rheumatism, fever, headache, toothache and as tonic
Withania somnifera (L.)	Asthma, cough
Xylocarpus granatum k.o.koening	Dysentery and breast tumours
Ziziphus nummularia (Burm.f.)	Skin diseases, cold, cough, biliousness
Zygophyllum simplex (L.)	Cardiac properties and applied in eye disease

Source: Gupta *et al*. (Editors), 2005. Ecology and Environmental Management: Issues and Research Needs, *Bulletin of the National Institute of Ecology.* **15**:81-97.

The 21st Century will possibly be the time of halophytic agriculture extension, due to various reasons particularly decline in resources of fresh water and avaibility of saline water, and civilizations are required to take advantage from vast saline soils and aquifers. Trillions of dollars are annually wasted from mildly saline fields while from new salt loving crops billions of dollars can be gained (Khan *et al*., 2009). Utilization of brackish and saline water resources for cultivation of cash crops as fuel, fiber, food, fodder and as medicines has been recommended for ever increasing population (Khan and Weber, 2008).

1.1. *Plant Description*

Different species (40) of genus Launeae of Family Compositeae (Asteraceae) growing in sandy, dry and saline habitats (Rozema and Flowers, 2008). In Pakistan, the genus is represented by 20 species mostly, with galactagogue, soporific, diuretic, wound healing, antipyretic and aperients properties (Ozenda, 2004). *L. nudicaulis* (L.) Hook.f. is important member of this genus. Its local name is Jangli Booti (Krishnamurth, 1969). Stems are few to many from base, often flexous, procumbent, or straggling, 15-60 cm long rarely branched below, radical leaves 5-20 cm, sessile, lobed, margins in older leaves beset with white cartilaginous minute teeth, heads 1-2 cm, across, often 5-10 cm each lateral dusters, yellow, rayed, achenes about 2 mm long, much shorter than the soft white pappus hairs (Figs. 1 and 2). Is known to be effective against itches, ulcers, cuts, swellings, bilious fever, eczema eruptions, cancers, microbial pathogens, insects and rheumatism. The leaves of *L. nudicaulis* (L.) Hook.f. are used as antipyretic in children, where as its latex is used to relieve constipation (Samia and Mohammad, 2000). In Pakistan it is found in waste places, vacant land and in cultivated fields (Riaz *et al*., 2012).

Fig. 1: Whole plant

Fig. 2: Root

2. MATERIALS AND METHOD

Collection of Plant Material

Plant at maturity was collected from premises of Karachi University identified by Prof. Dr. Rubina Dawar, Department of Botany, University of Karachi.

2.1. *Preparation of Extract*

Roots, stems and leaves of *L. nudicaulis* (L.) Hook.f. were separated, cleaned and weighed. Each morphological part (1000 g) was soaked in methanol (10 litres) with random shaking. Soaking vessels were cleaned and rinsed prior to use. After 20 days of soaking it was filtered through Whatmann filter paper No. 45. Buchi Rotavapor R-700 was used for evaporation of solvent. All the extracts were stored at 4°C for further phytochemical and *in-vitro* experimentations.

2.2. *Fluorescence Analysis*

Dried powder of all parts roots, stems and leaves, respectively were treated with different chemicals and organic reagents than subjected

to Ultra violet light and day light for florescence analysis. Observations were taken under long UV (365 nm), short UV (256 nm) and normal daylight.

2.3. *Physico-chemical Analysis*

Total ash value and moisture content of powdered samples of leaves, stems and roots of plant were determined.

2.4. *Total Ash Value*

Each powdered part (3 g) was placed in silica crucible to make sample carbon free and burnt them individually till red hot and then gradually increased flame until constant value obtained.

Total ash value (%) =
Weight of total Ash/Weight of sample × 100

2.5. *Moisture Content*

Powdered sample (1 g) was taken respectively in silica crucible and then placed in oven at 105°C for some time until constant weight of each part were obtained.

Moisture content (%) =
Loss in weight of sample/Weight of sample×100

2.6. *Sample Treatment for Elemental Analysis*

Each part of plant (100 g) was pulverized twice into fine powder state using Panasonic MX-J220P grinder, and each powdered sample was separately passed through 60 mesh sieve. Each powdered sample was kept in stoppered, clean, dry, and properly labeled glass vials.

2.7. *Chemicals*

Analytical grade nitric acid (HNO_3) and perchloric acid (70%) were used as reagent for wet digestion of samples; both were supplied from Fischer scientific. De-ionized water was used for preparation solution.

Sodium nitroprusside, phosphate buffer and sulfanilic acid reagent (B.D.H. Laboratory Supplies, UK), sodium hydroxide, sodium hypochlorite and thiourea (Sigma-Aldrich, USA) were used to determine urease inhibition activity.

2.8. *Digestion of Sample*

All three samples of plant were digested firstly by wet digestion method (Wazir *et al*., 2007). Powder 1 g of each sample was weighed on electrical balance of Sartorius CP 3245, than it was allowed to stand overnight in HNO_3 (10 ml) followed by careful heating of solutions on water bath (Gerhardh Schuttewasserbad SW20) till red nitrous oxide fumes are ceased. At room temperature it was allowed to cool. After cooling 70% $HCLO_4$ (4ml) was added followed by heating until small volume remained. Whatman filter paper No. 42 was used for filtration of remaining solutions. Filtrates were then transferred into volumetric flask (50 ml) and distilled water was used to make up the volume.

2.9. *Standards and Instrumentation for Elemental Analysis*

With reference to standard solutions the concentration of each element was estimated and final calculations were made according to weight of samples and dilutions made of original solutions. Fresh standard solutions for each metal were prepared in order to avoid any degradation and maintenance of quality. Certified atomic absorption stock standard solutions of each metal were used. Different concentrations of standard solutions were used for all heavy metals by diluting stock of 1000 ppm. Volumetric flasks (Pyrex) used were of 0.01% accuracy. For heavy metal detection of each morphological part Perkin Elmer USA Model#AAnalyst 700,

Atomic Absorption Spectrophotometer was used. Standard working parameters used are mentioned in Table 2.

Table 2: Working Parameters of Atomic Absorption Spectrophotometer

S.No.	Heavy metal	Wave length (nm)	Slit width (nm)
1.	Lead (Pb)	283.3	0.7
2.	Manganese (Mn)	279.5	0.7
3.	Magnesium (Mg)	285.2	0.7
4.	Cadmium (Cd)	228.8	0.7
5.	Zinc (Zn)	213.9	0.7
6.	Iron (Fe)	248.3	0.2
7.	Chromium (Cr)	357.9	0.7
8.	Cobalt (Co)	240.7	0.2
9.	Nickel (Ni)	232.0	0.2
10.	Copper (Cu)	324.8	0.7
11.	Arsenic (As)	193.7	0.7
12.	Sodium (Na)	589.0	0.2
13.	Calcium (Ca)	422.7	0.7

Fuel used: Air acetylene

2.9.1. *Urease Inhibition Activity*

Determination of urease inhibition activity of each extract was performed as described earlier (Elmer, 1991; Lateef *et al*., 2012). Reaction mixture was prepared using Urease Enzyme (Jack Bean) 25 µl and buffer 55 µl, containing 100 Mm urea and with test compound 5 µl incubated for 15 minutes at 30°C in 96-well plates. Indophenol's method was used to determine urease activity by measuring ammonia production. To each well 45 µl of phenol reagent (1% w/v phenol and 0.005% w/v sodium nitroprusside) and 75 µl alkali reagent (0.5% w/v Sodium Hydroxide and 0.1% Sodium hypochlorite). After 50 minutes increase in absorbance was measured using Elisa reader (Spectra Max plus 384 Molecular Device, USA). All reactions were performed in triplicate and final volume of each reaction was 200 µl. Percentage inhibitions were calculated using following formula:

Urease inhibition (%) =
100-(OD test well/OD control) 100

3. RESULTS AND DISCUSSION

The use of herbal drugs and medicinal plants in the treatment, management and cure of diseases gained interest and things returned to more "Natural" which demands more research on and better use of Herbal remedies. The consumer demands for Herbal drugs increased when they came to know that even the sophisticated synthetic drugs have undesirable adverse and side effects. *L. nudicaulis* (L.) Hook.f. is considered as important medicinal plant of genus Launaea due to its use in treatment of many diseases including constipation, bilious fever, children fever, skin itching, cuts, ulcers, swellings, conjunctivitis, suppuration of abscess, eczema, eruption, rheumatism and toothache (Weatherburn, 1967). The therapeutic background of *L. nudicaulis* (L.) Hook.f. prompted us for investigation of plant. Phytochemical investigation is important for discovery of therapeutically active agents and it also provide us new sources of economical therapeutically active materials. Fluorescence analysis is important tool for characterization of crude drug. Different chemical constituents are responsible for fluorescence in plant material. Chemicals present in plants show fluorescence, some in visible range some in ultra violet light and that are not fluorescent may often be

converted into fluorescent by treating with reagents.

Moisture content evaluation showed less chances of microbial degradation of drug during storage because of low values of moisture content which are 7.20, 10.0, and 10.30 for Roots stems and leaves respectively. Excessive moisture encourages yeast and fungal growth during storage and also may cause break down of major constituents by enzymatic activity. Accepted range for total ashe value was 22% which indicates that plant has normal complexes of organic and inorganic components (Farrukh and Itrat, 2013). In addition urease inhibition activity results for extracts of roots, stems and leaves are not significant. According to WHO *H. pylori* is recognized as class 1 carcinogen. For its suppression worldwide efforts are being made through application of different therapies including some natural remedies that are safer and cost effective (*British Pharmacopoeia,* 1980).

This research was conducted to determine both toxic and essential metals in three parts of plant. As a result of this high concentration of cobalt, chromium and calcium in stem while nickel, copper and arsenic was present in small amount. Roots contain iron and copper in high concentration and cadmium, calcium and cobalt in small amount. Leaves has small amount of chromium and cobalt with high quantities of calcium, cadmium, lead, arsenic, magnesium, manganese, zinc, and iron. Sodium was found in almost all parts of plant with some variations (Fig. 3).

3.1. *Phytochemical Screening*

Preliminary phytochemical screening of methanolic extract of roots showed the presence of alkaloids, carbohydrates, diterpenes, flavonoids, proteins, steroids, and saponin glycosides. Methanolic extract of stem showed the presence of alkaloids, carbohydrates, diterpenes, flavonoids, phenolic compounds, proteins, steroids, saponin glycosides, and tannins.

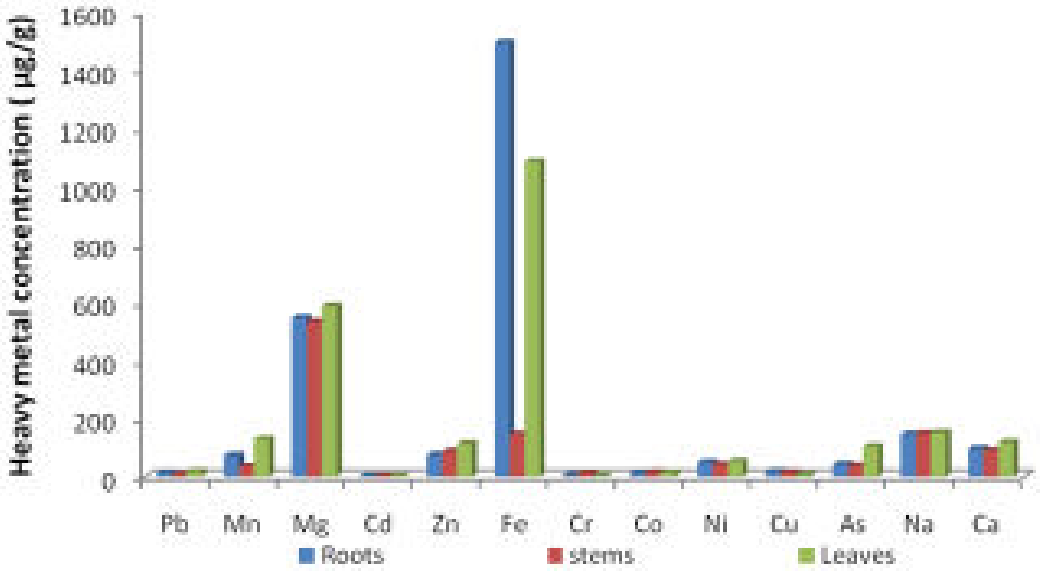

Fig. 3: Heavy metal concentration of root, stem and leaves extract of *Launeae nudicaulis* (L.) Hook.f.

While screening of leaves extract give positive test for presence of alkaloids, carbohydrates, diterpenes, flavonoids, phenolic compounds, proteins, steroids, saponin glycosides, and tannins. Presence of all phytochemicals in each morphological part is depicted in Table 3.

3.2. *Elemental Analysis*

Lead (Pb)

Lead maximum content 18.05 µg/g was found in leaves and minimum content was calculated in stems (8.25 µg/g) while roots contain 12.95 µg/g of lead

Manganese (Mn)

Leaves contain maximum manganese content (131 µg/g) and minimum amount was found in stems (35.3 µg/g).

Magesium (Mg)

589 µg/g of magnesium was found in leaves of *L. nudicaulis* (L.) Hook.f. which is higher than stems which contained 535.3 µg/g and roots which contained 548 µg/g.

Cadmium (Cd)

Leaves of *L. nudicaulis* (L.) Hook.f.

Table 3: Phytochemical screening of Leaves, Stem and Roots of *L. nudicaulis* (L.) Hook.f.

Phyto-Constituents	Test	Leaves	Stems	Roots
Proteins	Millon's	+	–	+
	Biuret's	+	–	+
	Ninhydrin's	+	–	+
Phenolic Compounds	$Fecl_3$	+	+	–
Steroids	Chloroform + H_2SO_4	+	+	+
	Chloroform + H_2SO_4 + Acetic acid	+	+	–
Saponin Glycosides	Froth formation	+	+	+
Carbohydrates	Benedict's	+	+	+
	Mollisch's	+	+	+
	Fehling's	+	+	+
	Barfoed's	–	–	–
Tannins	$FeCl_3$	+	+	–
	Gelatin	+	+	–
Flavonoids	Lead acetate	+	+	+
	Alkaline reagent's	+	+	+
Diterpenes	Ca–acetate	+	+	+
Terpenoids	Chloroform	–	–	–
Alkaloids	Dragendroff's	+	+	+
	Mayer's	+	+	+
	Wagner's	+	+	+

Present (+), Absent (–)

contained maximum Cadmium (2.15 µg/g) while minimum was found in roots (1.85 µg/g) and 2.05 µg/g was found in stems.

Zinc (Zn)

Zinc maximum amount was observed in leaves (114.5 µg/g) and minimum was observed in roots (76.75 µg/g) while stems contained 88.05 µg/g of zinc.

Iron (Fe)

L. nudicaulis (L.) Hook.f. is found to be rich in Iron. Roots contains maximum concentration of iron (1497 µg/g) and stems showed minimum concentration (145.75 µg/g) while leaves also contained high concentration of iron (1087 µg/g).

Chromium (Cr)

Chromium highest content was calculated in stems (12.05 µg/g) while leaves showed minimum concentration (2.77 µg/g) and roots contained 5.1 µg/g of chromium.

Cobalt (Co)

Highest concentration of cobalt (15.6 µg/g) was found in stems, leaves contain 14.5 µg/g and roots contain least concentration (13.2 µg/g) of cobalt.

Nickel (Ni)

Nickel (53.75 µg/g) was found in leaves and roots contained 50.55 µg/g while least amount was found in stems (42.1 µg/g).

Copper (Cu)

Maximum copper content (18.35 µg/g) was found in roots and minimum was found in leaves (6.7 µg/g) while stems contained 16.15 µg/g of copper.

Arsenic (As)

Leaves of *L. nudicaulis* (L.) Hook.f. contained maximum arsenic content (105 µg/g) than roots (42.725 µg/g) and least content was found in stems (36.6 µg/g).

Sodium (Na)

Sodium content (152.47 µg/g) found in leaves whereas roots and stem contained 146.33 µg/g and 148.833 µg/g sodium respectively.

Calcium (Ca)

Calcium highest content was found in leaves (119.2 µg/g) and least found in stems that is 91.71 µg/g where as roots contain 98.21 µg/g of calcium.

Table 4: Heavy Metal Concentration Roots, Stems and Leaves of *L. nudicaulis* (L.) Hook.f.

Heavy metals	Roots	Stems	Leaves
Pb	12.95	8.25	18.05
Mn	74.45	35.3	131
Mg	548	535.5	589
Cd	1.85	2.05	2.15
Zn	76.75	88.05	114.5
Fe	1497	145.75	1087
Cr	5.1	12.05	2.77
Co	13.2	15.6	14.5
Ni	50.55	42.1	53.75
Cu	18.35	16.15	6.7
As	42.725	36.6	105.9
Na	146.433	148.833	152.47
Ca	98.21	91.71	119.12

3.3. *Fluorescence Analysis*

Fluorescence Analysis of powdered roots stems and leaves of *L. nudicaulis* (L.) Hook.f. revealed different colors of fluorescence when treated with solvents and then these powders of plant were observed under day light and UV (254 nm and 366 nm). The observations are given in Tables 5, 6 and 7 for fluorescence analysis of roots, stems and leaves respectively.

Table 5: Florescence Analysis of Root Extract.

Solvents	Sun light		Short wave length (254 nm)		Long wave length (366 nm)	
	0 min	30 min	0 min	30 min	0 min	30 min
Dist. Water	Milky	Pale	Yellow	Pale	Brown	Brown
Methanol	Milky	Pale	Milky	Yellowish	Brown	Brown
HCl	Light brown	Light brown	Yellow	Yellowish	Brown	Blackish brown
H_2SO_4	Brown	Black	Brown	Black	Brown	Blackish brown
KOH	Pale	Pale	Yellow	Yellow	Brown	Brown
NaOH	Yellow	Yellow	Yellow	Yellow	Brown	Brownish black

Table 6: Florescence Analysis of Stem Extract

Solvents	Sun light		Short wave length (254 nm)		Long wave length (366 nm)	
	0 min	30 min	0 min	30 min	0 min	30 min
Dist. Water	Milky	Yellowish	Yellow	Yellow	Brown	Brownish black
Methanol	Light brown	Light brown	Yellow	Yellow	Brown	Brown
HCl	Light brown	Yellowish brown	Yellow	Yellow	Brown	Black
H_2SO_4	Light brown	Blackish	Yellow	Brown	Brown	Black
KOH	Pale	Brown	Yellow	Yellow	Brown	Black
NaOH	Pale	Brown	Yellow	Yellow	Brown	Black

Table 7: Florescence Analysis of Leaves Extract

Solvents	Sun light		Short wave length (254 nm)		Long wave length (366 nm)	
	0 min	30 min	0 min	30 min	0 min	30 min
Dist. Water	Light brown	Off white	Milky	Yellow	Brown	Dark brown
Methanol	Light brown	Light brown	Pale	Yellowish brown	Brown	Dark brown
HCl	Light brown	Pale	Green	Brownish yellow	Brown	Black
H_2SO_4	Light brown	Black green	Brownish brown	Blackish	Dark brown	Black
KOH	Pale	Yellow	Green	Yellow	Brown	Brownish black
NaOH	Yellow brown	Yellowish	Green	Yellow	Brown	Brownish black

3.4. *Physico-chemical Analysis*

Physico-chemical analysis is important parameter in determination of adulteration. Total Ash value and moisture content of *L. nudicaulis* (L.) Hook.f. is tabulated in Table 8.

Table 8: Physicochemical Evaluation of powdered Roots, Stems and Leaves of *Launeae nudicaulis* (L.) Hook.f.

Part Used	% w/w	
	Moisture content	Total ash value
Roots	7.20	13.45
Stems	10.0	10
Leaves	10.30	15.30

3.5. *Urease Inhibition Activity*

Urease inhibition activity of roots, Stems, and leaves of *L. nudicaulis* (L.) Hook.f. depicted in Fig. 4. It was compared with standard i.e. Acetohydroxamic acid was used as standard. The percentage of urease inhibition activity of standard Acetohydroxamic acid was 99.8% with IC_{50} 41.1±0.234. The Urease inhibitory effect produced by Root extract was found to be 14.3% and stem extract was 18.3% while leave extract showed 22.2% urease inhibitory effect. All these extracts showed inhibition less than 50% therefore due to its low efficacy IC_{50} values of extracts were not calculated. As a result of this it is stated that urease is an enzyme which is widely distributed in plants, microorganisms and animals which catalyzes the hydrolysis of urea to carbonium ion and ammonium ions and induce carbonate precipitation. This enzyme is present in plant to

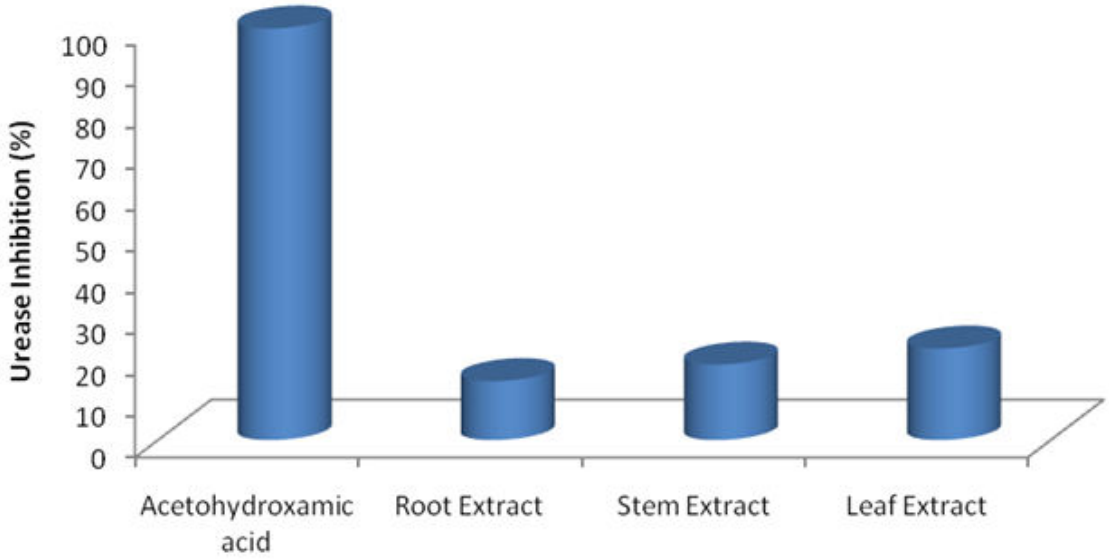

Fig. 4: Urease Inhibition activity of root, stem and leaves extract of *Launeae nudicaulis* (L.) Hook.f.

hydrolyze urea fertilizer (Dilrukshi RAN and Satoru Kawasaki, 2016). It is present in *L. nudicaulis* (L.) Hook.f. and helpful in biomineralization or cementery of soil and ultimately useful in increasing economic feasibility and to reduce the production of unwanted by-products. Low level of urease activity showed the plant i.e. *L. nudicaulis* (L.) Hook.f. has ability to maintain sustainability in order to reduce the production of CO_2 emission to regulate eco-friendly environment.

4. CONCLUSION

Increase in population and global climatic changes at alarming rate predicted to quite decrease in per capita viability of water within next quarter of century which leads to realization of requirements of top new avenues in supply of food and health securities. *L. nudicaulis* (L.) Hook.f. has been proved to be effective in treatment and management of various diseases. The present investigation adds to already existing knowledge of *L. nudicaulis* (L.) Hook.f. and will be useful for identification and authentication parameters of plant for safe human use as well as will also be beneficial for further research and study of plant. Preliminary phytochemical screening and Physico-chemical analysis of Roots, stems and leaves will be useful in order to standardize pharmaceutically and avoid any adulteration in the drug. Plant has negligible urease inhibition activity while knowledge of elemental concentration can further be used in study of utilization of this plant in different ailments and as food and fodder as it was not been performed before. High iron concentration present in roots and Leaves can be used in number of iron deficiency diseases. World wide it is one of the most prevalent micronutrient deficiency that directly effects formation and

functions of different organs of body, particularly brain. Low income countries have high prevalence of Iron deficiencies among its population especially due to poor diet and high level of infections. Natural sources of these micronutrients are not only affordable but also quite safe. More over such kind of flora may be proved as economically feasible and eco-friendly agents which help to control the soil for good agricultural attempt.

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Dietary Management of *Takayus al mebyadh* (Polycystic Ovarian Syndrome)

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Abstract

In Unani system of medicine the diet therapy (*Ilaj bil Ghiza*) is an important part for managing many diseases. Diet is one of the most important factors that keeps the *Akhlat* (humors) in equilibrium and ultimately maintains a balanced *mizaj* (temperament) on the other hand modern medicine also puts great emphasis on this aspect. PCOS (Polycystic ovarian syndrome)-insulin resistance-obesity are interconnected. The modification(s) in dietary patterns in PCOS patients improves metabolic and reproductive outcomes by reducing insulin resistance. No such article was there which accurately enlist appropriate food items for such patients. PCOS is due to *Sue Mizaj-Barid Ratab* (Mal temperament due to cold and moist). *Mizaj* of Foods with hot and dry are the best, while cold and moist are the worst. On the other hand food with low glycemic index are best while high glycemic index should be avoided with respect to the concept of modern medicine. This review article focuses on the most healthy and unhealthy food for PCOS patients with respect to the concepts of Unani and modern medicine and enlist appropriate food items for such patients with a distinct view.

Keywords

PCOS, Insulin resistance, Dietary pattern, *Mizaj*.

1. INTRODUCTION

Takayus al mebyadh (Polycystic ovarian syndrome, PCOS) is an endocrine disorder during reproductive age of women with high worldwide prevalence ranging from ~6.% to 10% (Norman *et al.,* 2007). Globally this syndrome has affected about 105 million women between 15 to 49 years of age (Sedighi *et al.,* 2015).

The most common characteristic features of the PCOS includes oligomenorrhoea, amenorrhoea, anovulation, infertility, hirsutism, acne and accelerated scalp hair loss. Its diagnosis requires the presence of two out of three of the following: a) oligo – or anovulation, b) clinical and/or biochemical signs of hyperandrogenism, and c) images of polycystic ovaries upon ultrasound. However other etiologies such as congenital adrenal hyperplasia, androgen secreting tumors and cushing's syndrome should be excluded (ESHRE and Group, 2004) which have been reviewed recently (Hameed *et al.,* 2016).

About 80% of women with PCOS are insulin resistant, thereby leading to hyperinsulinemia (Wright *et al.,* 2004). Insulin resistance (IR) at the receptor level is a pathological condition in which a cell, or a tissue requires high quantities of insulin to carry out normal physiological functions. The IR also enhances insulin secretion by pancreatic cells

and hence leads to hyperinsulinemia, while blood glucose remains normal. Once the response of pancreatic cells decreases, the levels of glucose increases and patient develops glucose intolerance or type II diabetes (De Leo V. *et al.,* 2003).

Insulin plays multiple role in PCOS as the high uncontrollable glucose levels may lead to insulin resistance-obesity association. The production of androgens from the ovary is largely dependent on two main hormones i.e insulin and leutinizing hormone (LH) along with the leptin levels which are raised under obese conditions (Conway and Jacobs, 1997). Moreover, insulin also inhibits the hepatic production of sex hormone-binding globulin (SHBG) protein (Jakubowicz and Nestler, 1997; Plymate *et al.,* 1988; Nestler *et al.,* 1991). Females suffering from PCOS have 25-30% chances of developing impaired glucose tolerance once they reach 30 years of age and 8% of them are more likely to acquire type 2 diabetes (Fauser *et al.,* 2012).

The incidence of obesity is 40%-60% in PCOS which is a major contributing factor in its pathogenesis and worsen the reproductive and metabolic complications. On the contrary, weight loss reduces insulin resistance, hyperandrogenism and most importantly restores ovulation. Thus limited caloric intake has been reported to reduce weight and IR in obese insulin resistant women (Hoeger, 2012). Since, IR appears to be a common factor in both PCOs and diabetes therefore same dietary restrictions will be applicable as it interferes with metabolic and hormonal abnormalities characteristic of the PCOS population (Anderson *et al.,* 1995; Pasquali *et al.,* 1989; Kiddy *et al.,* 1989). Therefore, modification(s) in dietary patterns improves metabolic and reproductive outcomes and it has been addressed in the present review.

1.1. *Concept of diet in Polycystic Ovarian Syndrome*

Two broad divisions regarding the concept of diet in PCOS is generally based on Unani medicine and Modern Medicine

In Unani system of medicine also referred as Eastern medicine (coined by Hakim Mohammed Said) the importance of diet has been documented since ancient times which can be validated by Hippocrates famous quote "Let food be thy medicine, and medicine be thy food" and is popular as *Ilaj bil ghiza* (dietotherapy). Egyptian, Roman, Greeks such as Buqrat (Hippocrates, 460-327 B.C.), Arastu (Aristotle 384-377 B.C.), Celus (53 B.C.-7 A.D.), Jalinoos (Galen, 130-200 A.D.) were great pioneers in dietetics/dietotherapy. They all emphasized the importance of diet during health and diseases. However, this concept gained more popularity during the time of our beloved Prophet Mohammed (ﷺ) (570-632 A.D.). Later, Bu Ali Ibn Sina (Avicenna, 989-1036 A.D.) realizing the importance of diet in maintaining the state of health complied a renowened book *Qulyat-e-Qanoon* (*Canon of Medicine*), remained a classical medical text book for many centuries in Europe and Arab world (updated edition Ibn Sina, 1998). Nowadays, the study of how food, or its particular components, can contribute to the long-term treatment of chronic ailments, is referred as dietotherapy. Indeed it has a long history, and stems from Zhou Dynasty, 1000 B.C., Zhang Ji, a distinguished physician in the Han dynasty who realized the action of dietotherapy and its impact on the patients well-being (Zaman, 2015).

The state of health is under the direct influence of lifestyle factors including diet. The equilibrium in *Akhlat* (humours) and *miza*j (temperament) is balanced by a combination of *Asbab-e-Sitta Zarooriyah* (six essential factors). The six essential factors (also called

lifestyle factors) are: i) ambient air, ii) food and drink, iii) physical activity and rest, iv) emotions and feelings, v) sleep and wakefulness, vi) retention of fluids and evacuation of wastes. *Mizaj* is one of the basic concept of Unani system of medicine. It maintains the ideal state of health and any alteration in it will cause *Sue Mizaj* (Mal temperament) which if stays may lead to diseased conditions one such example is of excessive intake of food and lack of physical activity which may result in obesity due to transformation of *mizaj* from normal to cold (Ibn Sina, 1998; Kabiruddin, 1930). Buqrat emphasized on proper dietary habits mainly to avoid overeating and practice *Qaylula* regularly (rest after consuming food). Furthermore, Jalinoos also recommended four important facts about diet: a) Time of the food b) Type of the food c) Quantity of the food d) *mizaj* of the food (Tabri, 2010). The classification different *mizaj* of food is described in Table 1.

Diet is one of the six essential factors which can modify the *mizaj* of an individual, e.g in PCOS patients it is deviated from balanced state to cold and moist state, high fat mass indicate the cold temperament as in case of PCOS patient (Ansari *et al.,* 2014).

The reproductive features of PCOS were noted by Buqrat in the 5th century B.C (Hanson, 1975) has been associated with *Sue Mizaj-Barid Ratab* (Excess of coldness and moisture) which is caused by qualitative and quantitative disturbances in the equilibrium of *akhlat* causing

Table 1: Classification of Intensity of *Mizaj* (Temperament) on the Basis of Degree After Food Consumption

S.No.	Intensity of *Mizaj* (temperament)	Effects on the human body (duration, hours)
1.	Zero degree *Motadil* (Balanced)	No effect or change in the body
2.	First degree (*Darjah awwal*)	Lasts for less than 4 hours
3.	Second degree (*Darjah doem*)	Last for more than 8 hours
4.	Third degree (*Darjah soem*)	Last for more than 18 hours and produces prominent noticeable and tolerable effects without harming the body
5.	Fourth degree (*Darjah chaharum*)	Intense effect immediately after 30 min to 1 hr and endangers one's life. All toxic/poisons are included with hot and dry effect

The *mizaj* (Temperament) are: Sanguine, choleric, phelgmatic and melancholic.
The degree of *mizaj* (zero to four) represents its intensity and duration of effect after food consumption. Hamdani, (1980), Qureshi, (2006), Siddiqi, (2006,) Dunlop, (1968).

excessive production of *Balgham* (phlegm) resulting in chronic anovulation ((Khan, 1983 and Tabri, 2010).

According to Unani physician Razi *mizaj* of the obese person becomes *Barid* (cold) and in such condition, the *Haar Yabis(* hot and dry), diet,drug and exercise are most suitable to reverse the conditions, however, cold and moist diet should be avoided (Razi., 1997).The good and worst foods with respect to their *mizaj* are listed in Table 2**.** The importance of diet has also been also reviewed by (Rizwani, 2011).

Table 2: Best and Worst Diet for Polycystic Ovarian Syndrome According to the Quality of *Mizaj*

S.No.	Best diet (Hot and Dry) for PCOS	GI	Worst diet (Cold and Moist) for PCOS	GI
1.	Chicken, fish, prawns, all small bird meat	0	Radish	0
2.	Eggs	0	Cucumber	15
3.	Mustard oil	0	Soya beans	18
4.	Red and green pepper	0	Milk full fat	27
5.	Chilli sauce	0	Buttermilk	31
6.	Cashew	0	Lettuce	32
7.	Hazel nuts	0	Okra	32
8.	Onions	15	Pear	38
9.	Lemon	20	Kiwi fruit	53
10.	Chick peas	28	Brown rice	55
11.	Garlic	30	Ice cream	61
12.	Bitter melon	32	Figs	61
13.	Grapes	46	Carrots	62

Glycemic index (GI) is a relative ranking of carbohydrate in foods according to how they affect blood glucose levels and the insulin secretion in the body is arranged in ascending order.

The temperament of PCOS is phlegmatic (Cold and Moist) and the best diet for them is Hot and Dry representing the opposite quality of food (Choleric temperament).

The worst diet for PCOS is Cold and Moist representing quality of food itself (Phelgmatic temperament).

While in modern medicine, according to World Health Organization (WHO), in 60% of the population lifestyle factors play a significant role on the quality of life and health of (Ziglio *et al.,* 2004). Life style modifications are safe option for the management of PCOS while diet is a trivial risk factor responsible for causing chronic diseases (Rahman *et al.,* 2012). Lifestyle strategies without rapid weight loss results in improved insulin sensitivity, reduction in central fat, and restoration of ovulation in overweight infertile women with PCOS (Huber *et al.,* 1999).

1.2. *Classification of Diet*

There are many types of diets, which is not possible to cover in this review however, some of the most important diets have been addressed and classified such as:

Low glycemic index diet, Low carbohydrate and high protein diet, Mono-unsaturated Fatty Acids, PUFA-rich diets, Saturated fatty acids and Hypocaloric diet.

1.2.1. *Low Glycemic Index (GI) Diet*

Glycemic Index (GI) is a method for classifying carbohydrate rich foods according to their effect on postprandial glycaemia that was introduced in 1980s (Markovic *et al.,* 1998). It involves "ranking of carbohydrates on a scale from 0 to 100 according to the raised blood sugar levels after eating" (Foster *et al.,* 2002). A low-GI diet can improve IR as well as many of its metabolic consequences (Wolever, 1992). On the contrary a high-GI diet has been reported to worsen postprandial IR by raising the blood sugar levels (Brynes *et al.,* 2003). Therefore consumption of foods with low GI value is highly favourable.

On the other hand, high-GI foods are rapidly digested but causes wide fluctuations in blood sugar levels, while low-GI foods are slowly absorbed and, therefore, produce gradual rise in blood glucose and insulin levels. According to numerous studies reports a direct relationship between low-GI diets and improved IR, particularly when compared to caloric restriction alone. In one study of post-menopausal women with elevated testosterone the dietary changes led to an increase in SHBG accompanied by a significant reduction in testosterone, waist-to-hip ratio, total cholesterol, fasting blood glucose and serum insulin (Sieri *et al.,* 2010). Low-GI diets are also associated with a lower incidence of type-2 diabetes mellitus, cardiovascular disease, metabolic syndrome, and endometrial cancer (Brand *et al.,* 2009). A low calorie and low GI diet helps in reducing the risks of PCOS complication in comparison with other diets (Egan *et al.,* 2011). Tables 3.1 and 3.2 shows food ingredients that are good or worse for PCOS with respect to Gl values which are grouped into Low (55 or less), Medium (56-69) and High (70 or more).

In case of foods with low GI reported to increase satiety, reduce hunger or lower subsequent voluntary food intake while high-GI foods are associated with increased appetite and higher energy intake (Ludwig, 2000; Roberts, 2000).

Both the quantity (per serving) and quality (nature or source) of carbohydrate influences the glycemic response. The GI is concerned with the quality of carbohydrate while, for glycemic load (GL) the quantity of the overall glycemic effect of a portion of food is important (Salmeron *et al.,* 1997). Thus higher GL is linked with elevated glucose levels in blood due to insulinogenic effect of the food. The long-term consumption of a diet with a relatively high GL (adjusted for total energy) is associated with an increased risk of type 2 diabetes and coronary heart disease (Liu *et al.,* 2000). GL is

Table 3.1a: Diet (Fruit) Suitable for Polycystic Ovarian Syndrome Women

S.No.	Food item(Fruits)	GI	GL	(*Mizaj*) Temperament
1.	Rasberries	0	0	Cold and Dry
2.	Cherries	22	3	Cold and Dry
3.	Apple (Juice)	38 (41)	15 (12)	Cold and Dry
4.	Pear	38	4	Cold and Moist
5.	Plums	39	5	Cold and Dry
6.	Strawberries	40	1	Cold and Dry
7.	Orange (Juice)	42 (50)	5 (12)	Cold and Dry
8.	Peaches	42	5	Hot and moist
9.	Grapefruit	46	8	Cold and Dry
10.	Grapes	46	8	Hot and Dry
11.	Mango	51	8	Hot and Moist
12.	Pineapple	51	7	Cold and dry
13.	Banana	52	12	Hot and Moist
14.	Kiwi fruit	53	6	Cold and Moist

Serving/g = 120 (250 ml)

Table 3.1b: Diet (Vegetables) Suitable for Polycystic Ovarian Syndrome Women

S.No.	Vegetables	GI	GL	(*Mizaj*) Temperament
1.	Egg plant	15	0	Cold and Dry
2.	Cucumber	—	—	Cold and Moist
3.	Onion			Hot and Dry
4.	Cabbage	45	—	Cold and Dry
5.	Lettuce	32	1	Cold and Moist
6.	Okra	32	0	Cold and Moist
7.	Radish	0	0	Cold and Moist
8.	Spinach			Hot and Moist
9.	Garlic	30	—	Hot and dry
10.	Lemon/juice	20	—	Cold and dry
11.	Bitter melon	32	1	Hot and dry

Serving/g = 100

Table 3.1c: Diet (Legume/grains) Suitable for Polycystic Ovarian Syndrome Women

S.No.	Legumes/grains	GI	GL	Serving per (g)	(*Mizaj*) Temperament
1.	Soya beans	18	1	150	Cold and Moist
2.	Kidney beans	18	7		Cold and Dry
3.	Pigeon peas	22	4		Cold and Dry
4.	Red lentil (Masoor dal) boiled	26	5		Cold and Dry
5.	Chick peas	28	8		Hot and Dry
6.	Lentils	29	18		Cold and Dry
7.	Green lentils	30	5		Cold and Dry
8.	Mung beans boiled	31	5		Cold and Dry
9.	Black eyed beans	42	13		Cold and Dry
10.	Barley	43	16		Cold and Dry
11.	Brown rice	55	18		Cold and Moist
12.	Wheat bread	53	11	20	Hot and Moist
13.	Green peas	54	4	80	Cold and Dry
14.	Oat bran	55	3	10	Hot and Moist

Table 3.1d: Diet (Dairy and Meat Products) Suitable for Polycystic Ovarian Syndrome Women

S.No.	Dairy and meat products	GI	Serving per (ml) or (g)	(*Mizaj*) Temperament
1.	Buttermilk	31	100 ml	Cold and Moist
2.	Cottage cheese	27	100	Hot and Moist
3.	Milk full fat	27	250ml	Cold and Moist
4.	Yogurt	36	200	Cold and Dry
5.	Egg	0	—	Hot and Dry
6.	Beef	0	100	Cold and Dry
7.	Chicken	0		Hot and Dry
8.	Lamb and mutton meat	0		Hot and Moist
9.	Fish	0	—	Cold and Dry

Glycemic index (GI) of different food item has been arranged in ascending order and they reduce the risk of PCOS because it produces gradual rise in blood glucose levels and improve insulin resistance. Foster *et al.,* (2002), Bhikha and Kragolsen, (2006).

Table 3.1c: Diet (Legume/grains) Suitable for Polycystic Ovarian Syndrome Women

S.No.	Legumes/grains	GI	GL	Serving per (g)	(*Mizaj*) Temperament
1.	Soya beans	18	1	150	Cold and Moist
2.	Kidney beans	18	7		Cold and Dry
3.	Pigeon peas	22	4		Cold and Dry
4.	Red lentil (Masoor dal) boiled	26	5		Cold and Dry
5.	Chick peas	28	8		Hot and Dry
6.	Lentils	29	18		Cold and Dry
7.	Green lentils	30	5		Cold and Dry
8.	Mung beans boiled	31	5		Cold and Dry
9.	Black eyed beans	42	13		Cold and Dry
10.	Barley	43	16		Cold and Dry
11.	Brown rice	55	18		Cold and Moist
12.	Wheat bread	53	11	20	Hot and Moist
13.	Green peas	54	4	80	Cold and Dry
14.	Oat bran	55	3	10	Hot and Moist

Table 3.1d: Diet (Dairy and Meat Products) Suitable for Polycystic Ovarian Syndrome Women

S.No.	Dairy and meat products	GI	Serving per (ml) or (g)	(*Mizaj*) Temperament
1.	Buttermilk	31	100 ml	Cold and Moist
2.	Cottage cheese	27	100	Hot and Moist
3.	Milk full fat	27	250 ml	Cold and Moist
4.	Yogurt	36	200	Cold and Dry
5.	Egg	0	—	Hot and Dry
6.	Beef	0	100	Cold and Dry
7.	Chicken	0		Hot and Dry
8.	Lamb and mutton meat	0		Hot and Moist
9.	Fish	0	—	Cold and Dry

Glycemic index (GI) of different food item has been arranged in ascending order and they reduce the risk of PCOS because it produces gradual rise in blood glucose levels and improve insulin resistance. Foster *et al.,* (2002), Bhikha and Kragolsen, (2006).

characterized into three categories (Foster, 2002) *viz* Low (0 to 10), Medium (11 to 19) and High (20 and over).

1.2.2. *Low Carbohydrate and High Protein Diet*

A 2 weeks clinical trial study on low carbohydrate and high-protein diet exhibited improvement in the glucose, insulin, and lipid profiles of type 2 diabetes (O'Dea *et al.,*1989). On the other hand a high carbohydrate diet (60%) for 15 days significantly increased mean very low-density lipoprotein cholesterol (VLDL-C) concentrations while significantly decreasing mean high density lipoprotein cholesterol (HDL-C) concentrations (Coulston *et al.,* 1987).

A number of studies in women with PCOS, overweight and obese subjects, as well as those with hyperinsulinaemia and type 2 diabetes have failed to show significant long-term benefits of a high-protein diet on weight loss (Moran *et al.,* 2003; Stamets *et al.,* 2004; Stern *et al.,* 2004).

Some of the diet which are high in protein are as follows chicken, prawns, all birds meat (hot and dry) beef, fish, crab (cold and dry), lamb, mutton (hot and moist).

1.2.3. *Monounsaturated Fatty Acids*

Monounsaturated Fatty Acids (MUFAs) are distinguished from the other fatty acid on the basis of having only 1 unsaturation in structure. In contrast, polyunsaturated fatty acids (PUFAs) containing 2 or more unsaturation in structure, and Saturated fatty acids (SFAs) are the straight chain structures (Lichtenstein, 1997). The most common monounsaturated fatty acids (MUFA) in daily nutrition is oleic acid, followed by palmitoleic acid, and vaccenic acid. Moreover, oleic acid represents the top most MUFA provided in the diet (~90% of all MUFA) (Connor *et al.,* 1997). While studies comparing high-monounsaturated fat with high carbohydrate diets have found a high-monounsaturated-fat diet results in reduced triacylglycerols and increased HDL-cholesterol, there is little evidence of the benefits of such diets for improving IR (Garg, 1998). However, one study of 162 healthy individuals, randomised to a high SAFs or high MUFA diet for 3 months, found that a high MUFA diet significantly decreased IR (Vessby, *et al.,* 1999). High MUFA and low carbohydrate diets are reported to be favorable while high carbohydrate diets are harmful. It was observed that a diet with high MUFA showed improvement in the serum concentrations of glucose, insulin, high-density lipoprotein, cholesterol and triglycerides (Markovic *et al.,* 1998; Tong *et al.,* 2002; Kelley *et al.,* 2004; Parillo *et al.,* 1992; Garg *et al.,* 1988; Garg *et al.,* 1994) (Table 4). Enlists the diet rich in MUFA and this shows that fats are mostly hot in *mizaj* with either moisture or dryness. Foods with hot and dry temperament are usually recommend by Unani medicine for PCOS patients.

1.2.4. *Polyunsaturated Fatty Acid*

Polyunsaturated fatty acid rich diets (PUFA) may not be as helpful as MUFA-rich diets. One study in women with PCOS found that an increased intake of PUFA resulted in a significant increase in fasting glucose levels but no change in insulin levels, blood lipids, testosterone or sex hormone-binding globulin levels (Kasim *et al.,* 2004).

1.2.5. *Saturated Fatty Acids*

Foods high in SFAs include fast foods, processed foods, high-fat dairy products and red meat are associated with metabolic syndrome. Diets rich in saturated fatty acids induce IR *in vitro* and *in vivo.* While animal studies demonstrate that a diet high in fat,

Table 4: Foods Rich in Monounsaturated and Polyunsaturated Fatty Acids

S.No.	*Mizaj*	Food ingredients	GI	MUFAs (%)	PUFA (%)
1.	Hot and moist	Almonds	10	66.2	25.9
2.	Hot and dry	Mustard oil	0	64.3	23.1
3.	Hot and Dry	Cashew	22	60.4	19
4.	Cold and Dry	Peanut	14	52.8	33.6
5.	Hot and Moist	Sunflower oil	0	48.3	38.1
6.	Hot and dry	Hazel nut	15	45.6	7.9
7.	Hot and Moist	Sunflower seed	20	40.2	50.2
8.	Hot and Moist	Mayonnaise	50	23	61
9.	Hot and Dry	Walnut	20	14.4	75.8

Foods with high MUFAs Monounsaturated fatty acids diet significantly decreased insulin resistance.
Per serving 100 g.
USDA, Agricultural Research Service. USDA Food Composition Databases. Available from https://ndb.nal.usda.gov/ndb/

particularly saturated fat, may lead to IR, human intervention studies investigating changes in dietary fat intake have been inconclusive, possibly due to their short duration and inadequate sample size (Riccadi and Rivellese, 2000). An increased risk of developing type 2 diabetes has also been associated with the consumption of higher-fat diets (Tsunehara *et al.,* 1991 and Colditz *et al.,* 1992). Some studies also indicate that a high-fat diet is more harmful in those who are inactive, supporting the importance of the role of exercise in managing IR (Mayer-Davis *et al.,* 1997).One such example is margarine which have cold and moist *mizaj* which is in line with the Unani concept also.

1.2.6. *Hypocaloric Diet*

The hypocaloric, low GI diet promotes a decrease in body mass index, percentage of body fat and improved oocyte development and pregnancy rate. (Becker *et al.,* 2015).

A major dietary change involves decreased intake of high GI carbohydrates and it is substituted with low GI foods and high fiber. Fats should be restricted equal or less than 30% of total calories with a low proportion of saturated fat (Moran *et al.,* 2013; Farshchi *et al.,* 2007). Monosaturated and polyunsaturated oils which provide a mixture of omega 3-fatty acids has been found to improve the lipid profile promote insulin sensitivity and according to several studies a diet with low calorie can lead to rapid weight reduction that can improve hyperandrogenemia, insulin resistance, and menstrual cycle (Pasquali *et al.,* 1989; Kiddy *et al.,* 1989; Holte *et al.,* 1995). The following food items are hypocaloric such as

pickled cucumber, lettuce, cucumber, celery, cabbage, radish, vinegar, tomato, bell pepper, spinach, coriander, cauliflower, eggplant and pumpkin.

2. DISCUSSION AND CONCLUSION

Diet is an important factor in controlling the fate of many diseases which is evident by both Unani and modern medicine. Incidence of PCOS is increasing worldwide and synthetic drugs have failed to provide a satisfactory management so the world is diverting towards nature, diet and herbs which are an effective and reliable approach for its management. In Unani system the *mizaj* of the individual and the food should be in balance to maintain a state of health and if any disease occurs due to a phlegmatic humor in PCOS and also obesity is a contributing factor and both are due to *Sue mizaj barid* so managing this factor by bringing balance to the *mizaj* will improve the condition of such patients, so a diet of the same *mizaj* which feeds this humor should be avoided and opposite *mizaj* diet of hot and dry should be consumed. Although there is no specific diet plan for PCOS in Unani or modern medicine the aim of this paper was to list some of the most suitable and unsuitable foods for PCOS with respect to both the system of medicines. Carbohydrates have an overall quality of moistness, fats have heat with degree of moistness. Proteins have an overall quality of dryness which are also favored by modern evidence based studies. There was some similarity in the list of foods which should be avoided or consumed .The best diet according to Unani system is Hot and dry and worst diet for such patients is cold and moist. Most of the foods with hot and dry *mizaj* were common in both list and the *mizaj* of food to be avoided was cold and moist which was very common between both the systems which was in line with the concept of the Unani scholars. Insulin resistance has not been mentioned specifically in the Unani literature but a correlation is proposed by us to relate the *mizaj* of diabetes type 2 and PCOS because both have a common pathogenesis that is IR so the diet and management strategies for diabetes are going to to be healthy also for PCOS.

The aim of this review is to provide a detailed list of food items which will help the physicians to better manage the conditions of PCOS patients with diet.

Author's Contribution and Declaration

The authors declare that there is no conflict of interest.

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Therapeutic Evaluation of Unani Pharmacopoeial Formulations for *Hisat-e-Kulyah* (Nephrolithiasis)

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Abstract

Nephrolithiasis is one of the most common health hazards worldwide. Despite advance technology of lithotripsy the management of renal calculi remains a challenge. Most of the Indian patients prefer alternative medicine rather undergoing painful surgery. The present paper deals with case study in which a 33 years old male patient of bilateral multiple renal calculi was treated with Unani pharmacopoeial medicine: *Qurs Kaknaj, Qurs Kushta Hajrul Yahood, Arq Kasni* and *Sharbat-e-Buzoori Motadil* as oral medication. Patient showed promising result in subsequent investigations and finally recovered from renal calculi without undergoing surgery.

Keywords

Nephrolithiasis, Diuretic, Litholytic, Lithotriptic, Nephroprotective.

1. INTRODUCTION

The term *Hisat-e-Kulyah* (Nephrolithiasis) is referred to as stone formation in the kidney. According to Ibn Sina (Avicenna) the stone is formed in the kidney by *quvat-e-fayelah* (active power) with comparatively raised or temperament than the normal kidney, and *maaddat-ul-hisat* (lithic matter) a viscous and sticky substance, may be either phlegm or viscous blood or pus. When expulsive power of kidney weakens due to altered temperament, hot inflammation or ulcer, then inspite of excreting out they are retained in the kidney. Thus the lithic substance dried by the *quvat-e-fayelah* (active power) of kidney, form crystals and gradually converts into stone. Nephrolithiasis is one of the most common health hazards worldwide with a prevalence rate of approximately 15% and in India about 2.3% (Haslet *et al*., 1999). The "stone belt" occupies some parts of Maharashtra, Gujarat, Punjab, Haryana, Delhi and Rajasthan in India (http://www.dilipraja.com/stone.htm, 2013). Various exogenous and endogenous factors are blamed to produce stone such as hereditary, dietary and urinary constituents (Haslet *et al*., 1999). These stones create problems by blocking the flow of urine and cause intermittent, sudden onset, severe pain termed as renal colic when they move along the ureter, if neglected it can cause obstructive uropathy, sepsis, and may lead to renal failure (Hussain *et al*., 1996).

Despite enormous technological advances in minimally invasive therapy like extracorporeal shock wave lithotripsy (ESWL) and percutaneous endourological techniques the management of renal calculi remains a challenge. Due to the high cost and adverse

effects of surgery, it is necessary to adopt the alternative and safe treatment to avoid surgical procedure.

In Unani system of medicine, the recommended principles of management to control nephrolithiasis and to expel out the disintigrated stones includes use of plenty of fluid, *tafteet-e-hisat* (litholytic/lithotriptic), *idrar-e-baul* (diuresis), *tahleel-e-waram* (resolution) *dafey-e-tashannuj* (anti-spasmodic), along with *taqwiyat-e-gurdah* (nephroprotective) (Razi, 2002).

2. MATERIALS AND METHODS

2.1. *Case Study*

A 33 years old man having nephrolithiasis, visited Govt. Unani Dispensary, Bheemganj Mandi, Kota, Rajasthan, India, for treatment. He suffered from intermittent pain in left lumbar and suprapubic regions of abdomen radiating towards thigh and tip of penis, associated with burning micturition and multi steam of urination with foul smelling and reddish yellow color. About 10 years back, he had past hisotry of lithotripsy back for renal stone.

The diagnosis was confirmed by ultrasonography (USG) of abdomen and pelvic. Findings showed a single calculus of 16 mm at mid pole of right kidney and multiple calculi of 7-11 mm at calyces of left kidney.

The patient has been prescribed *Qurs Kaknaj* (two pills), *Qurs Kushta Hajrul Yahood* (one pill), *Arq Kasni* (50 ml.) and *Sharbat-e-Buzoori Motadil* (20 ml) with plain water twice a day on empty stomach orally. All advised Unani compound drugs are pharmacopeal, marketed from GMP certified company Hamdard and prepared according to *Bayaz-e-Kabeer,* Vol. 2. These are 1) *Qurs Kaknaj,* 2) *Qurs Kushta Hajrul Yahood,* 3) *Arq Kasni* (125 ml contains *Tukhm-e-Kasni, Cichorium intybus* (Sd.), 15.60 mg and Dst) and 4) *Sharbat-e-Buzoori Motadil* presented in Tables 1-3.

Table 1: Composition of *Qurs Kaknaj*

S.No.	Component	Plant	Quantity (mg)
1.	*Gond Safaid*	*Acacia arabica* (Gum)	198.0
2.	*Habb-e-Kaknaj*	*Physalis alkekengi* (Fr.)	99.0
3.	*Maghz-e-Kharbooza*	*Cucumis melo* (Kernel)	–
4.	*Maghz-e-Kaddu*	*Cucurbita moschata* (Kernel)	69.0
5.	*Tukhm-e-Khashkhash*	*Papaver somniferum* (Sd.)	58.5
6.	*Aslussoos*	*Glycyrrhiza glabra* (Rz.)	–
7.	*Behedana*	*Cydonia oblonga* (Sd.)	–
8.	*Nishasta Gandum*	*Triticum sativum* (Sd.)	–
9.	*Tukhm-e-Khatmi*	*Althaea officinalis* (Sd.)	–
10.	*Tukhm-e-Khubbazi*	*Malva sylvestris* (Sd.)	–
11.	*Tukhm-e-Khurfa*	*Portulaca oleracea* (Sd.)	49.5
12.	*Kateera*	*Cochlospemum religiosum* (Gum)	39.0

Composition of formulation with quantity.

Table 2: Composition of *Qurs Kushta Hajrul Yahood* (each 90 mg. contains)

S.No.	Component	Plant	Quantity (mg)
1.	*Shora Qalmi*	Potassium nitrate	187.00
2.	*Hajrul Yahood*	*Lapis lazuli*	93.50
3.	*Aab-e-Turab*	*Rafanus sativus* (Rt.)	1.496

Composition of formulation with quantity.

Table 3: Composition of *Sharbat-e-Buzoori Motadil* (each 25 ml. contains)

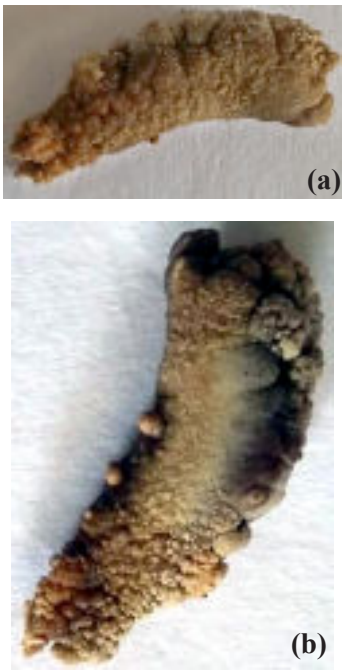
S.No.	Component	Plant	Quantity (mg)
1.	*Bekh-e-Kasni*	*Cichorium intybus* (Rt.)	4.32
2.	*Qand-e-Safed*	Sugar (Crystal)	24.2
3.	*Bekh-e-Badyan*	*Foeniculum vulgare* (Rt.)	–
4.	*Tukhm-e-Kakdi*	*Cucumis sativus* (Sd.)	–
5.	*Tukhm-e-Kasni*	*Cichorium intybus* (Sd.)	–
6.	*Tukhm-e-Kheera*	*Cucumis sativus* (Sd.)	–
7.	*Tukhm-e-Kharbooza*	*Cucumis melo* (Sd.)	2.01

Composition of formulation with quantity.

3. RESULTS AND DISCUSSION

The clinical response was excellent and significant as 8 mm calculus flushed out on 7th day of treatment (Figs. 1a and b). Ultra-sonography (USG) performed after 30 days, findings revealed single calculus of 5 mm at mid pole of right kidney and multiple calculi of 3-4 mm at calyces of left kidney. Further USG performed after 60 days from starting of treatment day did not detect any calculus. Thus Unani pharmacopoeial medicines used were safe, effective and prevented urinary super saturation of lithogenic substances. The beneficial actions of these pharmacopeal medicines can be attributed to the presence of complex spectrum of their ingredients having multiple mechanism actions including anti-inflammatory, antimicrobial, diuretic, antispasmodic, litholytic and lithotriptic activities.

Qurs Kaknaj has been described as diuretic (*idrar-e-baul*), litholytic (*mufattit-e-hisat*), lithotriptic (*mukhrij-e-hisat*), renal and urinary bladder wound healer (Zill-ur-Rahman, 1980). Its chief constituent *habb-e-kaknaj* (*Physalis alkekengi* fruit) is commonly used since ancient times in Unani medicine as diuretic, lithotriptic, anti-inflammatory and nephroprotective (Ali, 1999; Ghani, 1920; Aawan, 1993; Gufran *et al*., 2013). Main phytochemical constituents of *Physalis*



Figs. 1a and b: Excreted out *Hisat-e-Kulyah* (Nephrolithiasis)

alkekengi are alkaloids (Tropanes), flavonoids, sterols, fatty acids and amino acids (Sanchooli, 2011). The flavonoids are reported to have a role in analgesic action by targeting the prostaglandins and alkaloids are known for their ability to inhibit pain perception (Ibironke *et al*., 2007). It also possesses antispasmodic activity mainly *via* calcium influx blockade, partially through blocking adrenoceptors and nitric oxide synthesis (Mohammad *et al*., 2008).

Qurs Kushata Hajrul Yahood is useful as a diuretic and a lithotriptic (Zill-ur-Rahman, 1980). Its regular use prevents further formation of stone in kidney. It is specially given in pathological conditions of the urinary tract such as retention of urine, gonorrhea and urethral ulcer (Zill-ur-Rahman, 1980). *Hajrul Yahood Bhasma* is a rich source of Magnesium hydroxide $Mg(OH)_2$ which react with calcium oxalate calculus and forms magnesium oxalate soluble complex (Ali, 1999; Johansson *et al.* 1982). This process helps in disintegration of large calculi into the smaller particles. *Aab-e-Turab* (*Rafanus sativus* root juice) demonstrated for its anti-urolithiatic and diuretic activities (Vargas *et al*., 1999).

Arq Kasni has property to normalize the pungency of blood and bile; they are active cause for burning micturition (*hirqat-ul-baul*) (Zill-ur-Rahman, 1980). Its chief ingredient *Tukhm-e-Kasni* (*Cichorium intybus* seed) screened out as anti-microbial activity (Shaikh *et al.,* 2012). It is reported, the crude aqueous and organic seed extracts of *Cichorium intybus* were active against four pathogenic micro-organisms, namely, *Candida albicans Escherichia coli, Pseudomonas aeruginosa,* and *Staphylococcus aureus,* (Shaikh *et al.,* 2012). It is fact that the *Escherichia coli* is the most common cause (upto 85%) of *Tadiyah Majra-e-baul* (urinary tract infection) presented as burning micturition (*hirqat-ul-baul*) (Sood and Gupta, 2012).

Sharbat Buzuri Moatadil mainly indicated as diuretic (*mudirr-e-baul*) (Zill-ur-Rahman, 1980). It is claimed to be anti-urolithiatic agent preventing the recurrence stone formation by forming soluble calcium compound with citric acid. It also has alkalizing effect (Aziz *et al.,* 2011). Its chief ingredient *Bekh-e-Kasni* (*Cichorium intybus* root) possesses anti-inflammatory and nephro-protective activities (Cavin *et al.,* 2005; Shafaq and Tabassum, 2009). It is reported the ethyl acetate extract of *C. intybus* roots produced inhibition of prostaglandin E2 (PGE2) production in human colon carcinoma HT29 cells by inhibition of expression of cyclooxygenase-2 (COX-2) and direct inhibition of COX enzyme activity (Cavin *et al.,* 2005). The ameliorative effect of ethanolic extract of *C. intybus* was investigated using cisplatin induced nephrotoxicity on rats.

The extract reduced nephrotoxicity with no sign of toxicity (Shafaq and Tabassum, 2009). *Tukhm-e-Khiyarain* (*Cucumis sativus*) demonstrated anti-urolithiatic activity by hastening the process of dissolving the stones in kidney. It prevents oxalate induced lipid peroxidation and causes regeneration of renal epithelium (Janapareddi *et al.,* 2013).

Our study demonstrated that the pharmacopoeial Unani compound drugs are safe and effective in the treatment of *Hisat-e-Kuliyah* (Nephrolitiasis), by facilitating stone expulsion with significant improvement in symptoms associated with renal stones. Hence, to avoid surgery Unani formulations could be useful as an alternative therapy in *Hisat-e-Bauliyah* (Urolithiasis).

Conflict of Interest

The authors declare that there is no conflict of interest.

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Obituary

Zahida Bano

We announce, with deep sorrow, the passing of a quiet but strong pillar of Hamdard Pakistan: Zahida Bano Sahiba, Director of English Publications. إِنَّا لِلّٰهِ وَإِنَّا إِلَيْهِ رَاجِعُونَ (To Allah [s.w.t.] we belong and to Allah [s.w.t.] is our return).

Zahida Bano served Hamdard for almost four decades. She worked with Hakim Mohammed Said, Mrs Lily Anne D'Silva and Mrs Sadia Rashid, and formed bonds of friendship and trust, which she earned with her hard work and dedication to duty. Neither advancing years nor worsening health turned her away from her strong work ethics, which may have forced her to adjust her working hours, but did not allow her to compromise the quality of work or interfered with the job in hand. Though her physical step sometimes wavered and even caused her to fall, her professional step remained sure and firm. These traits, along with her honesty, simplicity and dignity, earned her the respect among senior and junior colleagues alike.

She handled much of the editing and all of the proof-reading of Hamdard's Felicitation Volumes honouring various world renowned scholars and scientists. This was also her well-undertaken responsibility for the journal *Hamdard Medicus* and occasionally *Hamdard Islamicus,* as well as four volumes of collection of essays of Hakim Mohammed Said.

In the month of October 2015, she suffered a heart attack, which left her too weak to continue her professional duties. A gradually worsening condition, caused her to breathe her last at about *'Isha* on 10 January 2017. She was buried in Karachi. May Allah rest her soul in peace (*Ameen*). Well educated, well read, exemplary worker, staunch friend: Zahida Bano, you are – and will be – much missed.

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Published by Hamdard University, Madinat al-Hikmah, Shahrah-e-Madinat al-Hikmah,
Muhammad Bin Qasim Avenue, Karachi-74600, Pakistan.
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	Price	
	Inland Pak.Rs.	**Foreign US$**
Annual	750/-	150/-
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	(inclusive of airmail charges)	

Printed by MAS Printers, Nazimabad, Karachi-74600, Pakistan.