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Review Article

PHYTOCHEMICAL INGREDIENTS AND PHARMACOLOGICAL PROPERTIES OF *BERGENIA CILIATA*

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ABSTRACT

Bergenia ciliata belongs to the family Saxifragaceae, commonly called as Pashaanbheda or Zakham-e-hayat. Traditionally, *Bergenia ciliata* is mainly used to treat kidney stones for a long time. The plant contains very high amount of bergenin in addition to other phytochemical constituents like afzelechin, catechin, beta-sitosterol, gallic acid, tannic acid, (-)-3-0-galloylepicatechin, (-)-3-0galloylcatechin, gallicin, etc. It has number of pharmacological properties such as antibacterial, anti-inflammatory, anticancer, anti-tussive, anti-diabetic, anti-lithotriptic, antidiabetic, antimicrobial, etc. Hence, there is an urgent need for documentation of such tribal species and help to find innovative ways to tap its efficiency used for human welfare in future. Although, some herbal formulations containing *B. ciliata* are available for therapeutic applications.

INTRODUCTION

Medicinal properties of a large number of botanicals and their healing components have been extracted and analyzed. In the 19th century, first therapeutically active compound morphine was isolated from opium plant (*Papaver somniferum*) followed by salicylic acid, a precursor of aspirin was originally derived from white willow bark and meadow sweet plant (Kinghorn, 2001; Samuelsson, 2004). At present about 25% of the drugs prescribed worldwide are from plants, 121 such active compounds being in current use. Out of the 252 drugs considered as basic and essential by the WHO. 11% are exclusively of plant origin and a significant number are synthetic ones obtained originally from natural precursors (Sameulsson, 2004). Various herbal formulations have shown high therapeutic efficacy in prevention and treatment of chronic disorders (Shankar *et al.,* 2007; Chaudhary *et al.,* 2010; Jennifer, 2014). The most of these formulations contain phytochemical ingredients having high antioxidant potential and free radical scavenging activity. Nature produces an array of antioxidants which prevents the production of free radicals and protection against oxidative damage induced by various therapeutic and environmental toxicants. These phytochemicals also boosting mammalian antioxidant and immune defense thus help in early recovering from chronic disorders like diabetes, hepatic and renal disorders, etc.

Bergenia has attracted many researchers attention due to its varied medicinal activities. Various studies on *Bergenia* have been undertaken particularly for evaluation of germplasm resources, identification and isolation of bioactive ingredients besides its therapeutic activities. *B. Ciliata* is very rich in bergenin and it owes many of the medicinal activities to the presence of bergenin (Singh *et al.,* 2007; Chauhan *et al.*, 2012). Different researchers have scientifically evaluated this plant for different pharmacological activities.

CHARACTERISTICS

The plant is known as Paashanbheda (Paashan = rockstone, bheda = piercing) in Hindi and rock-foil in English which itself indicates that the *Bergenia* plants grow between rocks and appears to break them which bestows them with lithotriptic property. It consists about 30 genera and 580 species worldwide. It is found in Afghanistan, South Tibet and Bhutan. In India it is found in Himalayas, Arunachal Pradesh and other eastern states, and Jammu and Kashmir. It is considered as a miracle herb because it is used to cure several diseases viz., gastrointestinal problems, kidney stones, malaria etc. It is called Junteyenah in Arabian, Patharkuchi in Assamese, Patharchuri in Bengali. Pashaanbheda in Gujarati and Zakhm-e-hayat in urdu.

The plant *Bergenia ciliate* belongs to family Saxifragaceae, genus *Bergentia* and species *ciliata*. This family comprises of 30 genera and 580 species, mostly distributed in the cold and temperate Himalayas and Central and Eastern Asia between 4000 to 12000 feet. The genus *Bergenia* comprises about 6 species distributed in temperate Himalayas and central and Eastern Asia. Hooker (1888) has reported three species of *Bergenia* from India in the Flora of British India. Wehmer (1948) also reported three species of *Bergenia* from India in The Wealth of India. The salient botanical features of the genus *Bergenia* are: species of this genus are perennial succulent herbs upto 50 cm tall, distributed in temperate Himalayan region (from Kashmir to Nepal) from 2000-2700 m. above sea level in and around the Murreee area (Ruby *et al.*, 2012). Rhizomes woody, covered with leaf bases. Flowers are white, pink or

purplish in terminal cymes. Fruit is a conical capsule having minute seeds (Kumar and Tyagi, 2012).

TRADITIONAL USES

The roots of *Bergenia* has been considered to enjoy all the useful attributes of Gentirna root and has been regarded as demulcent and deobstruent, relieves pain in ribs and chest due to excessive cold humors, acts as effective diuretic and emmenagogue, lithotroptic and dissolves kidney's and bladder stones and relieves obstructions or toxic waste products, which remain in the alimentary canal, and urinary excretory system. The infusion is considered to be more active than root in asthma, bronchitis, epilepsy and spasmodic affections and to relieve flatulent colic in children. Root is effective to cambat chronic venereal diseases (Usmanghani *et al.,* 1997). *B. ciliata* rhizome extracts have proven anti-bacterial and anti-tussive properties (Rajbhandari *et al.* 2007: Islam *et al.*, 2002a). *Bergenia ciliata* is used in the traditional medicine of Asian as well as other countries. In Nepal, one tea spoonful of the juice of dried rhizome of *B. ciliata* along with an equal amount of honey has been taken orally 2-3 times a day by post-partum women against the digestive disorders as carminative and tonic as well. Dried rhizome powder, however, was prescribed at least a week or longer for bearing healthy build up. It is also used orally for anthelmintic.

PHYTOCONSTITUENTS AND THERAPEUTIC POTENTIAL

The qualitative phytochemical screening of rhizome of *Bergenia ciliata* rhizome was shown to contain flavonoids, glycosides, sterols, terpenoids and saponins, while alkaloids were found to be absent (Khan and Kumar, 2016). The phytochemical investigation on the aerial parts and on the leaves have resulted in the isolation of hydroquinone (benzenoids) (Sticher *et al*, 1979) (+) afzelechin, (+) catechin, quercetin-3-O-β-D-xylopyranoside,quercetin-3-O-α-L-arabinofurano-side, eryodictiol-7-O-β-D-glucopyranoside, arbutin, 62-O-p- hydroxybenzoylar- butin, bergenin, 4-O-galloylbergenin, 11-O-galloylbergenin, p-hydroxybenzoic acid and protocatechuic acid. 62 -O-protocatechuoylarbutin, 11-O-p-hydroxybenzoylbergenin, 11-O-protocatechuoylbergenin and 62 -O-p-hydroxybenzoylparasorboside (Fujii *et al*, 1996). (-)-3-O-galloylepicatechin and (-)-3-O-galloylcatechin (Bhandari *et al*, 2008). Extract of rhizomes were showed the presence of bergenin, phenolic compounds leucocyanidin, gallic acid, methyl gallate, catechin and polymeric tannin (Dixit and Srivastava, 1989). The rhizomes also yielded a new lactone compound Paashanolactone and confirmed the presence of (+)-catechin, (+)-catechin-3-gallate 11-O-galloyl bergenin and sterols viz., sitoindoside, β-sitosterol and β-sitosterol-d- glucoside (Chandrareddy *et al*, 1998). Bergenin is major compound in the rhizome (0.6%). It is 4-methoxy-2-[(1S,2R,3S,4S,5R)-3,4,5,6-tetrahydro-3,4,5-trihydroxy-6-hydroxymethyl)-2H-pyran-2- yl]-α-resorcylic acid δ-lactone monohydrate. It is a C-glucoside of 4-O-methylgallic acid. The molecule is composed of three six-membered rings: an aromatic ring, a glucopyranose ring and an annellated δ-lactone ring. The glucopyranose ring exhibits only small deviations from an ideal chair conformation. The annellated δ-lactone ring possesses the expected half-chair conformation. There is one intra- and six intermolecular hydrogen bonds which form an extensive hydrogen-bonding network within the crystal.

PHARMACOLOGICAL ACTIONS

Ruby *et al.* (2012) reported various pharmacological activities of Bergenia species in experimental animals. *B. ligulata* having antilithic activity, diuretic activity, anti-bradykinin activity, antiviral activity, antipyretic activity, antibacterial, anti inflammatory, hepatoprotective activity, insecticidal activity, α- glucosidase activity are due to presence of its constituents like; β-sitosterol, tannic acid, stigmesterol, gallic acid, bergenin, (+)- afzelechin, (+)-afzelechin, (+)-afzelechintetraccetate, (+)-5,7,4'-trimethoxyafzelechin, (+)-tetramethoxyazelechin, (+)-3-acetyl-5,7,4'-trimethoxyafzelechin. *B. ciliata* having antitussive, antiulcer, antioxidant, antibacterial, hypoglycemic, toxicological activity as it contains tannic acid, gallic acid, glucose, metarbin, albumen, bergenin, (+)-catechin, gallicin and gallic acid. *B. stracheyi,* shows DPPH radical scavenging activity, antimicrobial, xanthine oxidase inhibitory activities and also used in arthritis. The main chemical constituent of the species is bergenin

Singh *et al.* (2007) determined bergenin and gallic acid in different Bergenia species simultaneously. A simple, highly precise method has been established for the simultaneous determination of the bioactive molecules bergenin and gallic acid in three different species of *Bergenia-B. ligulata; B. ciliata* and *B. stracheyi*. The assay combines separation and quantitative estimation of the analytes on silica gel 60GF 254 HPTLC plates with visualization under UV light and scanning at 260 nm. This study has enabled simultaneous analysis of bergenin and gallic acid in all three species. A highly precise RP-HPLC method coupled with photodiode-array was used for determination of these compounds in the *Bergenia* species. *B. ciliata* and *B. stracheyi* were found to contain the most bergenin, 3.28 and 3.28%, respectively where as *B. ligulata* contained 2.42% bergenin.

Srivastava and Rawat (2008) showed botanical and phytochemical comparison of three Bergenia species: *B. ligulata, B. ciliata* and *B. stracheyi.* Botanical study of rhizomes revealed that *B. ciliata* has large

Table 1:
Phytoconstituents of *Bergenia ciliata* and their activity

Compounds name	Class of compound	Activity	References
Bergenin	Phenol	Antioxidant activity against ascorbic acid	Chauhan *et al.* 2013
Tannic acid	Phenol	Pharmaceutical application	Chauhan *et al.* 2012
Gallic acid	Phenol	Antifungal, antiviral, cytotoxicity, antioxidant	Pokhrel *et al.* 2014
Catechin	Phenol	Histidine decarboxylase inhibitor	Pokhrel *et al.* 2014
(-)-3-0-galloylcatechin	Phenol	-	Ruby *et al.* 2012
(-)-3-0-galloylepicatechin	Phenol	-	Ruby *et al.* 2012
Gallicin	Phenolic acid	-	Pokhral *et al.* 2014
Beta-sitosterol	Sterol	Inhibit cholesterol	Masood *et al.* 2006
Arbutin	Glycoside	Prevent the formation of melanin	Kumar and Tyagi 2013
Leucoanthocyanidin-4-(2-galloyl)	Glucoside	-	Pokheral *et al.* 2014
(+)-afzelechin	Flavanoid	-	Chauhan *et al.* 2012
Camphor	Terpenoid	Antinociceptive, antispasmodic, antimicrobial	Gyawali 2014
Clucoside	Terpenoid	-	Chauhan *et al.* 2013
2-pentanone	VOCs	-	Gyawali and Kim, 2012
Hexanal	Organic compound	Antifungal	Gyawali, 2014
2-methyl-1-propanol	Organic compound	-	Gyawali and Kim, 2012
Acetic acid	Organic compound	Therapeutic activity	Gyawali and Kim, 2012
Heptanol	alcohol	-	Han *et al,* 1998
2-ethyl hexanol	alcohol	-	Chauhan *et al.* 2013
3-pentanol	alcohol	-	Gyawali 2014
2-pentanol	alcohol	-	Gyawali and Kim 2012
Octanol	alcohol	-	Gyawali and Kim 2012
Pentanol	alcohol	Antibacterial and antifungal	Gyawali and Kim 2012
Heptanal	alcohol	-	Peana *et al.* 2002
2,4-Dimethyl-3-pentanone	VOCs	-	Gyawali, 2014
Limonene	Terpene	Antiseptic and chemotherapeutic agent	Moreau *et al.*, 2002
Linalool	Terpene alcohol	Food additives and shows bioactivity	Peana *et al.,* 2002
3-methyl-4-hexen-2-one	-	-	Khan *et al.*, 2014
2-nitropropane	Nitro compound	Shows hepatotoxicity	Masood *et al.*, 2006
Hexanol	Isomeric organic compound	Therapeutic activity	Kumar and Tyagi, 2013
2,4-hexadienal	-	Therapeutic activity and sequiterpene	Khan *et al.,* 2014
Alpha-terpinol	-	Myorelaxant and antispasmodic effects	Gyawali and Kim, 2012
Pentanoic acid	Carboxylic acid	Fragrant causing agent	Gyawali and Kim, 2012
2,4-nonadienal	-		BiomutagenicGyawali and Kim, 2012
Hexanoic acid	Carboxylic acid	Antifungal activity	Gyawali, 2014
Hexalactone	-	-	Bhandari *et al.,* 2008
Isobutyrophenone	-	-	Dhalwal *et al.* 2008
5,6-dihydro-2-pyranone	-	-	Rajkumar *et al.*. 2010
Decanoic acid	Fatty acid	Antimicrobial and antifungal activity	Sinha *et al.,* 2001
Nonanoic acid	Fatty acid	Herbicidal activity	Fujii *et al.*, 1996
2-methyl butanoic acid	Fatty acid	-	Chauhan *et al.*, 2012
Methyl nonanoate	-	Antimicrobial and antifungal activity	Bhandari *et al.,* 2008
Methyl cinnamate	Cinnamic acid	Antimicrobial activity	Mazhar-ul-islam *et al.,* 2002
Beta-phellandrene	Terpene	Used in fragrances	Byahatti *et al.,* 2010
[E]-4-hepten-2-one	Ketones	Used in fragrances	Gyawali, 2014
Quercetin-3-o-beta-D-xylopyranosides	Flavanoids	-	Kumar and Tyagi, 2013
Quercetin-3-alpha-Larbinofuranoxide glycosides	flavanoids	-	Kumar and Tyagi, 2013
Tannin, saponins, coumarins	-	Antimicrobial activity	Shankar *et al.,* 2016
Phenolic and flavanoid	-	Antimicrobial and antifungal activity	Agnihotri *et al.,* 2015; Pereira *et al.*, 2016
Terpenoids and cardiac glycoside	-	Phytotoxic activity	Ullan *et al.,* 2015
Epicatechin, catechin	-	Anti0inflammatory, antioxidant activity	Srivastava *et al.,* 2015
Terpenoid	-	-	Ahmad *et al.,* 2016
Bergenin, p-hydroxy-benzoyl bergenin	-	antioxidant	Sadat *et al.*, 2015
Alkaloid, saponin, glycoside, tannin and phenol, reducing sugars, flavanoid	-	-	Sadat *et al.*, 2015

number of starch grains; *B. ligulata* has maximum calcium oxalate crystals while *B. stracheyi* has a lesser amount of starch grains. Physicochemical studies showed that *B. stracheyi* had highest percentage of all physicochemical parameters (total ash 15.8, alcohol and water soluble extractives 13.83 and 16.83, sugar 5.5 and tannins 7.86), except starch and acid insoluble ash, which were highest in *B. ciliata*.

Dharmender *et al* (2010) carried out simultaneous quantification of bergenin, catechin, gallicin and gallic acid and quantification of β-sitosterol using HPTLC from *Bergenia ciliata* (Haw.) sternb. Bhat (2017) studied bioprospection of *Bergenia ciliata* for its antioxidant, antidiabetic, hepato- and nephro- protective effects. Histopathological studies revealed regeneration of β-cells of islets of Langerhans with daily administration of extract in a dose dependent manner.

Antitussive activity

Rajbhandari *et al.* (2007) studied the anti tussive activity of the methanolic extract of the rhizome of *Bergenia ciliata* in a cough model induced by sulphur dioxide gas in mice and observed the extract exhibiting significant anti-tussive activity in a dose-dependent manner as compared with control. Kumar *et al.* (2001) also reported the effect of *B. ciliata* roots for its bronchodilator action and reported that aqueous extract and chloroform fraction of *B. ciliata* roots exhibited significant relaxation of the isolated guinea pig tracheal strips and also inhibited the histamine and acetylcholine induced contractions on isolated tracheal strips. It also significantly delayed the onset time of the histamine and acetylcholine induced bronchospasm in guinea pigs.

Sinha *et al* (2001) evaluated antitussive activity of *B. ciliata* rhizome extract in mice. The methanolic extract of the rhizome was used in cough model induced by sulphur dioxide gas in mice.

Antioxidant activity

Methanolic and aqueous *B. ciliata* rhizome extracts were found to possess antioxidant activity, including free radical scavenging activity and lipid peroxidation inhibition potential (Islam *et al.*, 2002b). The antioxidant activity of methanolic and aqueous extracts of *B. ciliata* was also evaluated by Rajkumar *et al.* (2010) and observed both extracts having free radical scavenging effect that might prevent oxidative damages to biomolecules. Sajad *et al.* (2010) has reported the extracts of *B. ligulata* having free radical scavenging activity. Saha *et al.* (2014) has reported the oral co-administration of hydro-methanolic extract with ethylene glycol prevented the lipid peroxidation and decreasing the activities of antioxidant enzymes in kidneys, thereby suggesting its antioxidant activity.

Roselli *et al.* (2012) investigated the antioxidant, anti-α-glucosidase and anticholinesterase activity of the leaves and rhizomatous extract of *B. cordifolia* and reported that rhizome extract showed a higher degree of 1,1-diphenyl-2- picrylhydrazyl radical scavenging and anti-α-glucosidase activity than reference compounds (rutin and acarbose respectively). The study revealed that previously unknown minor constituents from the plant, (+)-catechin 3-O-gallate, (+)-catechin 3,5-di-O-gallate and 1,2,4,6-tetra-O-galloylb- D-glucopyranoside, were the radical scavenging and anti-α-glucosidase principles. These compounds as well as the crude extracts were weak acetylcholinesterase inhibitors, suggesting a higher degree of selectivity against α-glucosidase enzyme. Major constituents of the plant, bergenin and arbutin, were poor radical scavengers and enzyme inhibitors in comparison with the minor constituents.

Ruby *et al.* (2015) reported evaluated *in-vitro* antioxidant and hemorrhoidal potential of hydroethanolic leaf extracts of *B. ciliata, B. ligulata* and *B. stracheyi* using ten antioxidant assays and reported that absorbance for ferric thiocyanate assay and thiobarbituric acid (TBA) assay was 0.058 nm and 0.082 nm.

Purohit *et al.* (2017) investigated *in-vitro* antioxidant activity of solvent extracts of *B. stracheyi* by determining total phenolic content (TPC), DPPH radical scavenging method, superoxide anion radical scavenging assay and total antioxidant activity.

Nazir *et al.* (2011) determined antioxidant and antimicrobial activities of bergenin and its derivatives obtained from *B. stracheyi* by chemo-enzymatic synthesis. The studies revealed that biological activity of bergenin can be optimized by selective modification of its structure.

Bagul *et al.* (2003) studied phytochemical evaluation and free radical scavenging properties of rhizome of B.ciliata and found out that the methanolic extract showed good scavenging activity against DPPH (1,1-diphenyl-2-picryl hydrazyl) radical with an EC50 of 36.24μg/ml. the extract scavenged superoxide radical in a dose dependent manner with EC50 of 106.48μg/ml. Kumar *et al.* (2010) studied about the antioxidant properties methanolic and aqueous extracts of *B. ciliata* rhizome and found both the extracts to be free radical scavengers.

Antimicrobial activity

Islam *et al.* (2002a) evaluated antibacterial activity of ethanol, hexane, ethyl acetate, chloroform, butanol and aqueous extracts of roots and leaves of *B. ciliata* and observed that these extracts gave promising results against gram positive and gram negative bacteria viz. *Bacillus subtilis, Bacillus megaterium and P. aeruginosa.* Sajad *et al.* (2010) reported that various

fractions of *B ligulata* were also found to be antibacterial and inhibited *in vitro* cultures of *Escherichia coli, Bacillus subtilis* and *Staphylococcus auereus.* Similarly the root and leaf extract of *B. ciliata* was observed to be antifungal and was effective against *Microsporum canis*, *Pleuroetus oustreatus* and *Candida albicans*. Methanol and aqueous extract from rhizomes of *B. ligulata* inhibited *in-vitro* replication of influenza virus in a dose dependent manner thereby demonstrating its antiviral activity too (Rajbhandari *et al.*, 2001). The extract inhibited viral RNA synthesis and reduced viral peptide synthesis due to its active compound viz. condensed tannins present in it.

Adhikary *et al.* (2011) also found antimicrobial activity of number of plant extracts including *B. ciliata* and such activity was attributed to rich concentration of flavonoids and glycosides present in various analyzed plants. Methanolic extract of *B. ciliata* showed antimicrobial activity against *E. coli, Klebsiella sps* and *Serratia sps.* However, methanolic extract of *Astilbe rivularis* exhibited highest antibacterial effect against *E. coli* while each bacterial strain varies in its sensitivity. Bergenin has demonstrated Germicide activity against species *E. coli* and *Pseudomonas aeruginosa* and is also effective against many types of fungus by blocking a crucial enzyme called yeast alcohol dehydrogenase required for fermentation reactions. (Han *et al.*, 1998; Nazir *et al.*, 2007 and Zuo *et al.*, 2005)

Khan *et al* (2015) studied about antimicrobial activities of five compounds isolated from ethyl acetate fractions of *Bergenia ciliata* and found out that all compounds exhibited antimicrobial efficacy on the tested microorganisms that included two human Gram positive bacteria (*Staph auerus and Micrococcus luteus*) and four gram negative ones (E*. coli, P. aeruginosa, enterobacter cloacae, K. pneumoia*). MICs ranged from 0.156 to >10microgram/ml and MBCs ranged from 1.26 to 15 microgram/ml. compound 1 showed antifungal activity 7mm while 2 to 5 have moderate activity 5mm. Singh *et al* (2016) studied the antioxidant, antimicrobial and bioactive compounds of *Bergenia ciliata*.

Anti-neoplastic activity

The aqueous and methanolic extracts of *B. ciliata* rhizome were found to be promising to target tumors and to check neoplastic growth and malignancy (Islam *et al.*, 2002b). Rajkumar *et al.* 2011 evaluated the anti-neoplastic efficacies of the methanol and aqueous extracts of *B. ciliata* rhizome. The extracts were tested for their cytotoxicity on MDA-MB-435S (human breast carcinoma), Hep3B (human hepatocellular carcinoma) and PC-3 (human prostate cancer) cell lines by the XTT assay. Furthermore, the apoptotic inducing abilities of the extracts were analyzed by the cellular DNA fragmentation using ELISA. The results obtained suggested that the extracts bear anti-cancer metabolites and could be considered as a potential source for anti-cancer drug development.

Antifungal activity

Kumar and Tyagi (2013) evaluated the antifungal activity of different extracts of *Bergenia stracheyi* against six fungal species viz. *Alternaria alternate, Aspergillus niger, Colletotrichum gloeosporioides, Fusarium oxysporium, Ganoderma lucidum* and *Rhizoctonia solani* and reported that different extracts exhibit different extent of antifungal activity against all test fungi.

Cytoprotective activity

Kakub *et al* (2007) studied the cytoprotective effect of *B. ciliata* on gastric ulcers in rats. The study was done to evaluate its gastro-protective effects on ethanol/HCl, indomethacin and pylorus ligation-induced gastric ulcers in rats. Doses of 15, 30 and 60 mg/kg b/w of the aqueous and methanol extracts of the rhizome were administered 1 h after ulcerogenic treatment. The animals were killed 3 h later, their stomachs removed and the mean area of ulcer lesion was determined. The weight of mucus and gastric acidity were also measured. The aqueous extract decreased the ulcer lesion ($p < 0.05$) in all models to a greater extent than the methanol extract, but at the higher doses the effect was reduced.

Anti-inflammatory and antipyretic activity

Kumar *et al*. (2002) determined anti-inflammatory activity of aqueous extract of *B. ciliata* on carrageenin-induced paw oedema in rats. The extracts were found to be anti-inflammatory in activity and such activity was comparable to diclofenac sodium. Similarly, Sajad *et al.* (2010) also reported anti inflammatory activity that oral administration of the aqueous and ethanolic extract of *B. ligulata* showed anti-inflammatory activity. The antipyretic activity of *B. ligulata* in yeast induced fever in albino rats was evaluated by Singh *et al.* (2010) and it was reported that ethanolic extract from roots and rhizomes of *B. ligulata* decreased the induced fever in experimental animals.

Nazir *et al.* (2007) reported that bergenin effects anarchic or inflammation by balancing secretion of cellular messengers (cytokines) from pro-inflammatory and inhibitory cells of the immune system and also inhibits the release of inflammatory cytokines like interleukin-2 (IL-2), gamma interferon (IFN-β), and tumor necrosis factor-alpha (TNF-α). It was also found that bergenin promotes the release of anti-inflammatory messengers like IL-4 and IL-5 and stimulates the release of protective prostaglandins. Singh *et al.* (2009) evaluated antipyretic activities of ethanolic extract of root of *B. ciliata* in albino rats. Rectal temperature was recorded at different time intervals upto 3 hours after inducing pyrexia with brewer's yeast. It was reported that *B. ciliata* possessed antipyretic activity.

Anti-lithiatic activity and nephroprotective effect

The antilithiatic activity of *Bergenia* is due to its active compound bergenin which has high dissolving activity of calcium stones. Singla *et al.* (2014) observed the anti-lithiatic efficacy of bergenin isolated from the rhizome of *B. ligulata*. Bergenin treatment normalised the creatinine clearance in hypooxylouric rat model and was also effective in reducing oxidative stress markers like malondialdehyde and raised the decreased glutathione levels in kidneys. Byahatti *et al.* (2010) also reported that the phenolic compounds isolated from ethyl acetate fraction from leaves of *B. ciliata* resulted in high dissolution of both calcium phosphate stones and calcium oxalate stones.

Saha and Verma (2011) reported that *B. ciliata* extract/cystone along with ethylene glycol showed significant protective effect in body weight and organ weight with few stray areas of calcifications in glomeruli and also reported that *B. ciliata* extract has higher nephroprotective index than cystone at the same dose level. Saha *et al.* (2014) reported that co-administration of hydro-methanol extract of *B. ciliata* rhizomes with ethylene glycol in wistar rats caused restoration of calcium-oxalate and phosphate levels in urine and kidney. Similarly, Basher and Gilani, (2009) observed that hydro-methanolic extract of *B. ligulata* rhizome inhibited calcium oxalate (CaC_2O_4) crystal aggregation as well as crystal formation in the metastable solutions. Such extract also exhibited antioxidant effect, hypermagneseuric and diuretic effects in rats. Harsoliya *et al.* (2011) studied the effect of ethanolic extracts of *B. ligulata, Nigella sativa* and their combination on calcium oxalate urolithiasis in rats and observed that administration of ethanolic extract of either plant decreased the occurrence of kidney stone as compared to positive control group and combination of extracts as effective as the standard group.

Byahatti *et al* (2010) studied the effect of phenolic compounds from *B. ciliata* leaves on experimental kidney stones. The alcohol, butanol, ethyl acetate extracts and isolated phenolic compounds from *B. ciliata* were evaluated for their potential to dissolve experimentally prepared kidney stones- calcium oxalate and calcium phosphate by an in-vitro model. Phenolic compound P1, isolated from ethyl acetate fraction of the leaves demonstrated highest dissolution of both stones when compared to test extracts at 10mg concentration. Extract was more effective in dissolving calcium phosphate stones (67.74%) than oxalate (39.95%). Reference standard formulation cystone was found to be more effective (48.48%) when compared to compound P1.

Hypoglycemic activity

The hypoglycemic activity of the extracts of *B. ciliata* was evaluated for its potential of lowering the blood sugar lowering effects in streptozotocin (STZ) treated rats. The effects of various extracts on blood glucose revealed a significant decrease in blood glucose level (Islam *et al.*, 2002b). Saijyo *et al.* (2008) reported that the ethyl acetate extract of *B. ligulata* rhizome exhibited inhibitory effect on Q-glucosidase activity and therefore decreased blood sugar. The α-glucosidase inhibitor in *B. ligulata* is due to the presence of afzelechin. Singh *et al.* (2011) also evaluated the hypoglycemic effect of different extract (ether, chloroform, acetone and ethanol) of *B. ligulata* root in alloxan induced diabetic rats and reported that all the extracts possess hypoglycemic effect. However, maximum hypoglycemic effect was seen with acetone extract. Such studies conclude that *Bergenia spp.* plant has antidiabetic potential. Bhandari *et al* (2008) revealed the anti-diabetic potential of *B. ciliata* using an *in-vitro* model and isolated the active compounds from Pakhanbhed. Extraction and fractionation of the extract lead to the isolation of two active compounds, (-)-3-O-galloylepicatechin and (-)-3-O-galloylcatechin for the first time. These isolated compounds demonstrated significant dose dependent enzyme inhibitory activities against rat intestinal α-glucosidase and porcine pancreatic α-amylase.

Hepatoprotective effect

Singh *et al.* (2009) evaluated the hepatoprotective effect of *B. ligulata* in CCl_4 induced hepatotoxic male albino rat model and reported that ethanolic extract of roots decreased the elevated levels of liver biomarkers like SGPT, SGOT and total bilirubin in hepatotoxic male albino rats. The important bioactive phytoconstituents of bergenin of *Bergenia* species has demonstrated anti-hepatitis C virus (HCV) activity and weak anti-HIV activity *in-vitro*. However, it was not found effective at attacking HIV-I reverse transcriptase and HIV integrase (Han *et al.*, 1998; Nazir *et al.*, 2007 and Zuo *et al.*, 2005).

Anti-malarial effect

Walter and Bagai, (2016) reported that ethanolic leaf extract of *B ciliata* exhibited considerable inhibitory activity against both RKL-9 and MRC-2 strains of *P. falciparum* with IC_{50} of 6.4 μg/ml and <5 μg/ml respectively. The extract exhibited no toxicity against both cancer and normal cell lines with CC_{50} >1000 μg/ml and selectivity index (SI) >10. Maximum chemo-suppression of 74.45% and 91.96% was observed on day 7 at a concentration of 1000 mg/kg (repository activity) and 250 mg/kg (curative activity), respectively. 83.33% survival of mice was observed at 750 mg/kg while in all other ethanolic leaf extract of *B. ciliata* treated groups 50% survival was recorded on day 28 of study in the curative test. Hepatic function (SGOT, SGPT, ALP and bilirubin) and renal function biomarkers (creatinine

and urea) in serum were observed to be significantly lowered as compared to the infected control. Study concluded that ethanolic leaf extract of *B. ciliata* possessed considerable anti-malarial activity against both sensitive and resistant strains of *P. falciparum* and exhibits significant efficacy as a preventive and curative remedy against the disease without any side effects on hepatic and renal functions of the rodents.

Anti-biofilm activity

Liu *et al.* (2017) reported that the *B. crassifolia* (L.) leaf extracts showed inhibitory effects by decreasing viability of bacteria within the biofilm, as evidenced by the XTT assay, live/dead staining assay and LDH activity assay. There was decrease in the adherence property of *S. mutans* through inhibiting glucosyl-transferases to synthesize extracellular polysaccharides. In addition, the reduced quantity of extracellular polysaccharides and the inhibition of glucosyl-transferases were positively correlated with concentrations of test samples. Finally, the MTT assay showed that the extracts had no cytotoxicity against normal oral cells. Hence concluded that the extracts and sub-extracts of *B. crassifolia* leaves to be antimicrobial and could reduce extracellular polysaccharides synthesis by inhibiting activities of glucosyl-transferases to prevent bacterial adhesion and biofilm formation. Therefore, *B. crassifolia* leaves have potential to be developed as a drug to prevent and cure dental caries.

Antiviral activity

Rajbhandari *et al.* (2003) reported that hydromethanolic extract from rhizomes of *B. ligulata* inhibited in vitro the replication of influenza virus in a dose dependent manner and did not show virucidal activity at effective concentration. Pretreatment of cells with *B. ligulata* extract was shown to be most effective to prevent cell destruction. The extract inhibited viral RNA synthesis and reduced viral peptide synthesis at 10 mg/ml. The virus inhibitory effect is related to the presence of condensed tannins in the extract.

Anti-arthritis activity

Nazir *et al.* (2007) studied the immunomodulatory effect of bergenin and norbergenin against adjuvant-induced arthritis. Bergenin, a C-glycoside of 4-*O*methyl gallic acid, isolated from rhizomes of *B. stracheyi* (Saxifragaceae) and its *O*-demethylated derivative norbergenin, prepared from bergenin, are reported to show anti-arthritic activity through possible modulation of Th1/Th2 cytokine balance. Flow cytometric study showed that the oral administration of bergenin and norbergenin at doses of 5, 10, 20, 40 and 80 mg/kg per oral dose inhibit the production of pro-inflammatory Th1 cytokines (IL-2, IFN-α and TNF-α) while as potentiate anti-inflammatory Th2 cytokines (IL-4 and IL-5) in the peripheral blood of adjuvant-induced arthritic balb/c mice. This shows the potential Th1/Th2 cytokine balancing activity of bergenin and norbergenin which is strongly correlated with their anti-arthritic activity. At similar dose levels, the effect of 2 was found to be more than that of bergenin. The oral LD_{50} for bergenin and norbergenin was more than 2000 mg/kg body weight of the mice.

THERAPEUTIC FORMULATIONS

Indian Institute of Integrative Medicine (IIIM) Jammu has developed a bergenin based anti-inflammatory and anti-arthritic drug IIIM-160 (9.1% w/w), with dual activity of IL-6 inhibition and antinociception making it suitable for treating rheumatoid arthritis (Bharate *et al.*, 2019). Patel *et al* (2013) studied about wound healing activity of newly prepared polyherbal formulations (*Erythrina indica, Bergenia ciliata, Cissampelos pareira)* in excision and incision wound models in rats. Ointment treated group exhibited reduction in wound area on 20th day when compared to standard betadine ointment and controls with faster rate of epithelization. In the incision wound model, there was a significant increase in tensile strength observed in ointment treated group. In all cases, there was a progressive decrease in wound area with time, indicating an efficacy of the formulations in healing the induced wounds. Bharate *et al.* (2019) have prepared a gastro retentive sustained release formulations of *B. ciliata* for treating inflammatory diseases.

CONCLUSIONS

The present review detailed the information of *B. ciliata* and its therapeutic efficiency about the medicinal uses explained in medicinal systems. The phytochemical, pharmaceutical and biological investigation of *B. ciliata* reports the versatility and explains its diverse role. It is concluded that this medicinal herb has been used traditionally among the various communities across the tribal regions worldwide for various ailments. Additionally this herb is well known to treat kidney stones and kidney disorders by the traditional and local medical practitioners. In recent times, the old traditional practices are gradually declining very rapidly and are under risk due to rapid modernization. Hence, there is an urgent need for documentation of such tribal species and help to find innovative ways to u tap its efficiency used for human welfare in future.

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Research Article

AMELIORATIVE POTENTIAL OF CURCUMIN IN RATS EXPOSED TO METALLIC MIXTURE FOR 28 DAYS

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ABSTRACT

The present study was undertaken to evaluate the heavy metals (lead, arsenic, cadmium, mercury, iron and copper) mixture at 10x and 100x dose toxicity of environmentally realistic concentration in water bodies of Mathura district and its possible amelioration with curcumin @ 100 mg/kg body weight in male Wistar rats. Forty two rats were divided into seven groups- control, vehicle control, 10x, 100x, curcumin, 10x plus curcumin and 100x plus curcumin. The toxicity and its amelioration were considered on the basis of feed and water intake, body weight gain, absolute and relative organ weight and haemato-biochemical profiles. Heavy metals mixture resulted in a significant and progressive decrease in the feed and water intake resulting in dose dependent reduction in body weight gain at the two dose levels indicative of systemic toxicity which was substantially improved by co-administration of curcumin. The immunotoxic behavior of the mixture is indicated by a relative decline in the thymus weight. Dose–dependent increase in platelet count, mean corpuscular haemoglobin and haemotocrit and decrease in PCV and TLC was also observed when compared to control and vehicle control groups.However, ALT and AST were altered non-significantly.

Key words: Heavy metals, Metals mixture, Curcumin, Feed intake, Water intake

INTRODUCTION

Heavy metals adversely affect animals and human beings and causes subclinical or subtle effect which includes immunotoxicity, reproductive toxicity, nephrotoxicity, teratogenicity, neoplasms, cognitive and behavioral changes, production loss, population decline and ultimately serious economic losses over and above the health hazard. High levels of heavy metals in soil, water and feed/fodder has been reported in different parts of India (Rajaganapathy *et al.,* 2011). Metals like lead, cadmium, arsenic, mercury and iron affects biological function, endocrine system and growth of living beings and their bioaccumulation in animal tissues has evoked great concern for their residual toxicity.

There is a concern that several less studied complex exposures may have a large impact on animal and human health. The most common heavy metals (lead, arsenic, cadmium, mercury) have been recognized as highly toxic industrial and environmental pollutants which increases the possibility of human and animal exposure (Kokilavanti *et al.,* 2005). Although each metal has critical effect on specific organ or tissue, most metals affect multiple organs system (Goyer, 1996). Little is known about interactions among the various heavy metals during their co-exposure and their net combined toxic implications in humans as well as animals. The field of "mixtures toxicology" is emerging as an area of increasing scientific and regulatory focus. In a co-exposure situation, one compound alters the effect of other by altering its kinetics or dynamic behaviour.

Therefore, present study was planned to evaluate some toxicologically sensitive end points of general toxicity parameters and hemato-biochemical indices in male Wistar rats through sub-acute co-exposure of arsenic, lead, mercury and cadmium and essential macro-minerals like copper and iron for 28 days. Selection of the metals and concentrations used in this study was based on surveys conducted for residual concentration of these metals in the samples of feed/ fodder, water and soil collected from the adopted villages of our University (Zoheb *et al.,* 2015).

MATERIALS AND METHODS

A survey was conducted to measure the levels of Pb, As, Cd, Hg, Fe, and Cu in water samples. Seventy samples of water were collected from 7 villages (10 from each village) in and around Mathura district from different sources like hand pumps, tube-wells, river, ponds, tap water, and wells to which the animals have easy access. The concentrations of the selected metals in different samples were determined by Atomic Absorption Spectrometer (AAanalyst 400, Perkin Elmer USA). The highest concentration of different metals obtained in the samples were used for making mixture of all the six metals, which was then used in feeding adult male Wistar rats.

The mixture of all six metals was prepared at 10x and 100x dose for feeding experimental rats in vivo.

The concentration for mixture formulation of each metals was taken as 1/1000 of detected level and were Pb (0.188 ppm), As (0.038 ppb), Cd (0.016 ppb), Hg (0.011 ppb), Fe (1.792 ppm), and Cu (1.67 ppm) in deionized drinking water. Curcumin was dissolved in groundnut oil and given orally for 28 days, at a rate of 100 mg/kg body weight/day. The Research work was conducted at Department of Pharmacology & Toxicology, DUVASU, Mathura.

Experimental design

The study was conducted in adult male Wistar rats (250-270 g) procured from the Laboratory Animals Resources Section of Indian Veterinary Research Institute. Animals were maintained under standard management conditions. All the animals were quarantined for a period of at least seven days before beginning of the experiment. The animals were handled and the study was conducted in accordance with the Institute guidelines for the protection of animal welfare. Rats were divided into seven groups containing six/ group. Control rats were given deionized water (group I) and group II (vehicle control) were given groundnut oil. The animals of group III received curcumin at 100 mg/kg body wt. by oral gavage dissolved in groundnut oil. Rats of group IV, V, VI, and VII were administered with 10x, 100x metal mixtures, 10x metal mixture and curcumin, and 100x metal mixture plus curcumin, respectively daily for 28 days. Throughout the experimental period of 28 days, rats of all groups were observed for apparent signs of discomfort, physical abnormality, gait, diarrhea, and toxicity.

Clinical parameters

Throughout the experimental period of 28 days, rats of all the groups were closely observed for apparent signs of discomfort, physical abnormality, gait, diarrhoea or toxicity, if any, weekly body weight, weight gain percent, absolute and relative organs weight, feed and water intake were recorded.

Hematological parameters

After the exposure period of 28 days, blood was collected from retro-orbital plexus of rats with the help of glass capillary tubes, as per the procedure described by Sorg and Buckner (1964). Heparin was used as an anticoagulant and heparinised blood samples were centrifuged at 3000 rpm for 15 min. Plasma was separated, transferred into plastic vials and stored at -20^{0}C for further blood biochemical analysis. For determination of hematological parameters, blood was collected in heparinzed tubes and immediately used for determination of total erythrocyte count (TEC), total leukocyte count (TLC), differential leukocyte count (DLC) and packed cell volume (PCV) as described by Schalm *et al.* (1975) and haemoglobin (Hb) by cyanmet haemoglobin method using commercially available kit (Span Diagnostic Ltd) and the values expressed in gm/ dl.

Biochemical parameters

Total protein, albumin, creatinine, blood urea nitrogen, cholesterol, bilirubin, alanine aminotransferase (ALT), aspartate aminotransferase (AST) and glucose were estimated by using the commercially available kits (Span Diagnostic Ltd.) and autoanalyser (Erba, manheim).

Statistical analysis

Data have been expressed as mean ± SEM. Statistical analysis of data was performed using SPSS version 16 software. Data were analyzed by ANOVA and means were compared with Tukey's B multiple comparison post hoc test. A value of $p<0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

Environmental exposure is typical to multiple chemicals, little is known about interaction among the various heavy metals during their co-exposure through drinking water and their net combined toxic implication in humans and animals. Some metals like iron, copper and zinc are essential for proper functioning of organisms while lead, arsenic, cadmium and mercury are non-essential toxic elements. While several non-essential metals are known to be toxic at very low levels, metals that are biologically essential may also become hazardous at high level. Thus, potential interactions between toxic and essential metals are possible and could be critical modifiers of metal toxicity (Barbier *et al.*, 2005). Chronic low level exposure to toxic metals still continues to be a pertinent global problem. Little information seems to be available on the effect of a mixture of the most frequently occurring metals at environmentally realistic concentration in animals and humans. Number of studies on naturally occurring antioxidants like curcumin, ascorbic acid , α-tocopherol, melatonin are reported to mitigate the toxicity induced by single metal component such as arsenic, lead, cadmium and mercury induced toxicity in rats (Ghosh *et al.,* 2013; Sharma *et al.*, 2013). Therefore, it is of notable importance to study the mixture toxicity of metal (essential and non-essential) and its amelioration with curcumin.

Exposure of metals mixture @ 10x and 100x dose levels over a period of 28 days did not produce any apparent toxic clinical signs or mortality in rats. All the treated and untreated animals exhibited normal activities and behaviour. The change in body weight (g) and percent weight gain following 28 days of exposure at 10x and 100x dose and in combination with curcumin are presented in Table 1. Perusal of results revealed that, weekly body weight among different groups did not differ

Table 4:
Effects of metal mixture (10x and 100x), curcumin alone and in combination on hematological parameters following 28 days of continuous exposure

Groups		Parameters			
		Hb (g/dl)	TEC(10^6/μl)	TLC(10^3/μl)	PCV (%)
I	Control	14.00 ± 0.51[a]	7.14 ± 0.19[a]	11.07 ± 1.15[b]	46.15 ± 0.869[a]
II	Vehicle control	13.56 ± 0.58[a]	7.66 ± 0.17[a]	10.10 ± 0.80[ab]	50.57 ± 0.489[ab]
III	Curcumin	10.28 ± 0.66[a]	6.36 ± 0.94[a]	9.31 ± 1.17[ab]	51.16 ± 0.16[b]
IV	10x	14.07 ± 1.37[a]	8.68 ± 0.67[a]	10.55 ± 0.45[b]	51.66 ± 0.66[b]
V	100x	12.16 ± 1.39[a]	7.77 ± 0.62[a]	7.00 ± 0.49[a]	50.50 ± 1.47[ab]
VI	10x + Curcumin	11.31 ± 1.08[a]	6.89 ± 0.61[a]	9.49 ± 0.27[ab]	50.83 ± 1.72[ab]
VII	100x + Curcumin	13.98 ± 1.36[a]	8.92 ± 0.66[a]	10.67 ± 0.84[b]	50.83± 1.16[ab]

Values (mean ± SEM, n=6) bearing different superscripts in the same column vary significantly ($p<0.05$) in Tukey's B multiple comparison post hoc test.

Table 5:

Effects of metal mixture (10x and 100x), curcumin alone and in combination on differential leukocyte count following 28 days of continuous exposure.

Groups		Parameters				
		Neutrophils (%)	Lymphocyte (%)	Monocyte (%)	Eosinophils (%)	Basophils (%).
I	Control	26.02 ± 2.83[a]	44.30 ± 4.13[a]	7.37 ± 0.72[c]	1.61 ± 1.53[b]	0.00 ± 0.00[a]
II	Vehicle control	27.95 ± 2.29[ab]	45.51 ± 3.34[a]	6.75 ± 0.59[bc]	1.34 ± 1.07[ab]	0.00 ± 0.00[a]
III	Curcumin	27.95 ± 2.29[ab]	51.09 ± 5.02[a]	4.32 ± 0.42[a]	1.34 ± 1.07[ab]	0.00 ± 0.00[a]
IV	10x	37.79 ± 3.79[b]	56.06 ± 5.36[a]	5.28 ± 0.37[abc]	1.29 ± 1.14[a]	0.13 ± 0.02[b]
V	100x	38.44 ± 2.61[b]	57.40 ± 1.68[a]	4.50 ± 0.43[ab]	1.34 ± 1.28[ab]	0.26 ± 0.03[c]
VI	10x + CurCumin	33.12 ± 2.86[ab]	58.90 ± 4.18[a]	5.05 ± 0.44[abc]	1.27 ± 1.18[a]	0.00 ± 0.00[a]
VII	100x + Curcumin	36.39 ± 1.79[ab]	51.71 ± 2.16[a]	7.38 ± 0.76[c]	1.24 ± 1.09[a]	0.00 ± 0.00[a]

Values (mean ± SEM, n=6) bearing different superscripts in the same column vary significantly ($p<0.05$) in Tukey's B multiple comparison post hoc test.

Table 6:
Effects of metal mixture (10x and 100x), curcumin alone and in combination on hematological parameters following 28 days of continuous exposure.

Groups		Parameters				
		MCH (pg)	MCHC (g/dl)	PLT (x10^3/μl)	HCT (%)	MCV (fL)
I	Control	15.82 ± 0.54	33.69 ± 0.76	388.16 ± 40.98	24.37 ± 1.83	7.35± 0.13
II	Vehicle control	15.22 ± 0.25	30.90 ± 0.32	710.08 ± 33.85	43.94 ± 1.84	7.58± 0.20
III	Curcumin	15.90 ± 0.29	31.17 ± 0.60	387.05 ± 43.15	29.54 ± 2.67	8.82± 0.88
IV	10x	16.05 ± 0.19	30.58 ± 0.57	513.16 ± 34.48	40.60 ± 3.74	7.73± 0.13
V	100x	15.76 ± 0.75	30.33 ± 0.80	681.66 ± 73.81	44.31 ± 4.15	7.55 ± 0.19
VI	10x plus Curcumin	16.02 ± 0.33	31.38 ± 0.69	512.50 ± 52.69	36.71 ± 3.55	8.91± 0.49
VII	100x plus Curcumin	15.50 ± 0.824	30.46 ± 1.19	398.50 ± 40.23	42.48 ± 3.77	9.71±1.36

Values (mean ± SEM, n=6) bearing different superscripts in the same column vary significantly ($p<0.05$) in Tukey's multiple comparison post hoc test.

significantly however, slight decrease in the body weight of 100x intoxicated group of rats was observed on consecutive 7, 14, 21, and 28 days, compared to control, intoxicated and curcumin treated groups. After 28 days of exposure period, percent weight gain of 10x (10.39%) and 100x (6.32%) intoxicated groups were significantly ($p<0.05$) decreased compared to control (22.65%). Surprisingly, curcumin alone treated group also showed significant ($p<0.05$) reduction (16.40%) in gain, while, concurrent treatment with curcumin showed an increase in percent weight gain compared to 10x and 100x alone exposed group towards control group values.

The results of daily feed and water intake are summarised in Table 2. The results obtained showed significant ($p<0.05$) reduction in feed and water intake on first, second, third and fourth week as compared to control groups to their respective weeks after exposure at both doses whereas, significantly ($p<0.05$) increased feed and water intake towards control value and was also comparable with 10x and 100x alone treated groups. Interestingly, groups fed with curcumin alone @ 100 mg/kg body wt. showed significant decrease in feed and water intake as compared to control group. The change in data of absolute organ weight (g) and relative organ weight (g/100 gm body wt.) of liver, kidney, spleen, lung, brain, testes, heart, thymus and adrenals are given in Table 3. No significant change in the absolute and relative organ weights of liver, kidney, spleen, lung, brain, testes, heart and adrenals among different groups, however, 100x intoxicated group showed significant ($p<0.05$) decrease in the absolute and relative organ weight of thymus, whereas, mild to moderate increase in the

Table 1:
Effects of metal mixture (10x and 100x), curcumin alone and in combination on weekly body weight (g) and per cent body weight gain following 28 days of continuous exposure

Groups		Body weight (g) and body weight gain (%)					
		0 day	7thday	14th day	21st day	28th day	Weight gain (%)
I	Control	265.00 ± 4.73^{a}	284.33 ± 3.23 ^{a}	294.83 ± 3.86 ^{a}	314 ± 1.87 ^{a}	324.66 ± 2.18 ^{a}	22.65
II	Vehicle	265.00 ± 7.73 ^{a}	287.50 ± 8.44 ^{a}	303.66 ± 6.09 ^{a}	315.66 ± 5.81 ^{a}	323.50 ± 6.79 ^{a}	22.33
III	Curcumin	270.83 ± 10.06 ^{a}	287.33 ± 12.23 ^{a}	300.16 ± 13.76 ^{a}	310.00 ± 14.53 ^{a}	315.50 ± 13.37	16.40
IV	10x	272.50 ± 4.16 ^{a}	285.83 ± 4.31 ^{a}	290.66 ± 4.70 ^{a}	294.00 ± 4.86 ^{a}	300.8 3± 4.74 ^{a}	10.39
V	100x	263.16 ± 7.58 ^{a}	267.16 ± 8.13 ^{a}	271.66 ± 8.40 ^{a}	276.00 ± 8.67 ^{a}	280.00 ± 9.46 ^{a}	6.32
VI	10xplus Curcumin	272.66 ± 11.72 ^{a}	286.33 ± 14.15 ^{a}	294.00 ± 14.61 ^{a}	301.00 ± 14.60 ^{a}	309.33 ± 15.20 ^{a}	13.89
VII	100x plus curcumin	266.83 ± 9.43 ^{a}	273.50 ± 10.01 ^{a}	286.66 ± 10.30 ^{a}	294.83 ± 9.67 ^{a}	303.66 ± 9.04 ^{a}	13.93

Values (mean ± SEM, n=6) bearing different superscripts in the same column vary significantly (p<0.05) in Tukey's B multiple comparison post hoc test.

Table 2:
Effects of metal mixture (10x and 100x), curcumin alone and in combination on daily feed intake (g) following 28 days of continuous exposure.

Groups		Daily Feed intake (g) and Water intake (ml)							
		0-7thday		8-14th day		15-21th day		22-28th day	
		Feed	Water	Feed	Water	Feed	Water	Feed	Water
I	Control	153.14± 4.84^{d}	313.85±4.71^{f}	152.28± 3.94^{d}	315.85±4.93^{e}	158.57±3.42^{e}	317.85±6.72^{f}	161.57±4.91^{d}	336.71±7.15^{g}
II	Vehicle control	164.14± 2.34^{e}	281.14±2.58^{e}	167.4 2± 3.35^{e}	291.71±6.25^{d}	168.71±1.70^{f}	282.42±2.57^{e}	179.85±2.41^{e}	291.85±4.03^{f}
III	Curcumin	130.28 ±1.70^{ab}	218.57±2.18^{d}	134.28± 1.06^{b}	224.00±2.41^{c}	137.85±1.05^{c}	228.42±1.78^{d}	141.85±1.48^{c}	235.57±1.36^{d}
IV	10x	123.00 ±2.11^{a}	202.00±3.31^{c}	115.85±2.37^{a}	193.85±1.62^{b}	110.71±1.53^{b}	179.42±3.01^{b}	102.71±2.17^{b}	167.00±1.83^{b}
V	100x	120.85 ±2.58^{a}	157.85±3.24^{a}	110.28± 1.91^{a}	156.71±2.32^{a}	96.42±2.08^{a}	150.28±3.64^{a}	86.14 ±1.53^{a}	84.42±1.32^{a}
VI	10x plus Curcumin	139.57 ±1.39^{bc}	204.71±1.16^{c}	144.00±1.13^{cd}	218.42±2.64^{c}	149.42±1.78^{d}	229.28±2.11^{d}	157.14 ±1.63^{d}	251.57±4.11^{e}
VII	100x plus Curcumin	142.71 ±1.87^{c}	191.42±1.54^{b}	135.57±1.21^{bc}	190.28±2.00^{b}	145.85±1.77^{d}	200.00±3.17^{c}	158.57 ±2.05^{d}	217.57±2.52^{c}

Values (mean±SEM, n=6) bearing different superscripts in the same column vary significantly (p<0.05) in Tukey's B multiple comparison post hoc test.

Table 3:
Effects of metal mixture (10x and 100x), curcumin alone and in combination on absolute (g) and relative organ wt. (g/100g body wt.) following 28 days of continuous exposure.

OrganOrgans		Groups						
		Control	Vehicle control	Curcumin	10x	100x	10x + Curcumin	100x + Curcumin
Liver	Absolute weight	9.27±0.36^{a}	11.43±0.33 ^{a}	11.05±0.91 ^{a}	9.55±0.73 ^{a}	8.77±1.21 ^{a}	8.74±1.08 ^{a}	9.57±0 .92 ^{a}
	Relative weight	2.85±0.11 ^{a}	3.53±0.09 ^{a}	3.60±0.18 ^{a}	3.18±0.24 ^{a}	3.14±0.41 ^{a}	2.78±0.25 ^{a}	3.13±0.26 ^{a}
Kidney	Absolute weight	2.08±0.20 ^{a}	2.68±0.09 ^{a}	2.66±0.22 ^{a}	2.33±0.17 ^{a}	2.44±0.28 ^{a}	2.18±0.13 ^{a}	2.51±0.12 ^{a}
	Relative weight	0.64± 0.05 ^{a}	0.83±0.04 ^{a}	0.84±0.06 ^{a}	0.77±0.06 ^{a}	0.88±0.11 ^{a}	0.70±0.04 ^{a}	0.83±0.04 ^{a}
Spleen	Absolute weight	0.75±0.08 ^{a}	0.88±0.07 ^{a}	0.87±0.08 ^{a}	0.78±0.16 ^{a}	0.70±0.03 ^{a}	0.64±0.08 ^{a}	0.80±0.10 ^{a}
	Relative weight	0.23±0.02 ^{a}	0.27±0.02 ^{a}	0.27±0.02 ^{a}	0.25±0.05 ^{a}	0.25±0.01 ^{a}	0.20±0.02 ^{a}	0.26±0.02 ^{a}
Lung	Absolute weight	1.58±0.11 ^{a}	2.11±0.17 ^{a}	1.95±0.27 ^{a}	1.79±0.30 ^{a}	1.50±0.05 ^{a}	1.60±0.13 ^{a}	2.52±0.52 ^{a}
	Relative weight	0.48±0.03 ^{a}	0.65±0.06 ^{a}	0.61±0.04 ^{a}	0.59±0.09 ^{a}	0.54±0.03 ^{a}	0.52±0.05 ^{a}	0.81±0.15 ^{a}
Brain	Absolute weight	1.51±0.13 ^{ab}	1.79±0.05 ^{b}	1.30±0.03 ^{a}	1.71±0.05 ^{b}	1.51±0.11 ^{ab}	1.53±0.08 ^{ab}	1.60±0.07 ^{ab}
	Relative weight	0.46±0.04 ^{a}	0.55±0.02 ^{a}	0.42±0.01 ^{a}	0.57±0.02 ^{a}	0.54±0.05 ^{a}	0.50±0.04 ^{a}	0.53±0.03 ^{a}
Testes	Absolute weight	2.21±0.25 ^{ab}	2.89±0.14 ^{b}	2.89±0.11 ^{b}	2.49±0.10 ^{ab}	1.84±0.24 ^{a}	2.34±0.24 ^{ab}	2.42±0.33 ^{ab}
	Relative weight	0.68± 0.07 ^{a}	0.89±0.04 ^{a}	0.92±0.02 ^{a}	0.83±0.04 ^{a}	0.66±0.09 ^{a}	0.75±0.06 ^{a}	0.80±0.11 ^{a}
Heart	Absolute weight	1.04±0 .09 ^{a}	1.29±0.01 ^{a}	1.13±0.03 ^{a}	1.12±0.11 ^{a}	1.02±0.09 ^{a}	1.02±0.12 ^{a}	1.35±0.31 ^{a}
	Relative weight	0.32±0.02 ^{a}	0.39±0.00 ^{a}	0.36±0.00 ^{a}	0.37±0.03 ^{a}	0.36±0.03 ^{a}	0.32±0.02 ^{a}	0.45±0.10 ^{a}
Thymus	Absolute weight	0.28±0.04 ^{a}	0.19±0.00 ^{a}	0.24±0.01 ^{a}	0.25±0.03 ^{a}	0.50±0.11 ^{b}	0.25±0.02 ^{a}	0.17±0.02 ^{a}
	Relative weight	0.08±0.01 ^{a}	0.05±0.00 ^{a}	0.07±0.00 ^{a}	0.08±0.01 ^{a}	0.18±0.04 ^{b}	0.08±0.01 ^{a}	0.05±0.00 ^{a}
Adrenals	Absolute weight	0.05±0.01 ^{a}	0.06±0.00 ^{a}	0.03±0.00 ^{a}	0.08±0.03 ^{a}	0.10±0.04 ^{a}	0.05±0.00 ^{a}	0.04±0.00 ^{a}
	Relative weight	0.01±0.00 ^{a}	0.02±0.00 ^{a}	0.01±0.00 ^{a}	0.02±0.01 ^{a}	0.03±0.01 ^{a}	0.01±0.00 ^{a}	0.01±0.00 ^{a}

Values (mean ± SEM, n=6) bearing different superscripts in the same row vary significantly (p<0.05) in Tukey's B multiple comparison post hoc test.

absolute weights of testes exposed to 10x group was recorded. Compared to control, 10x mixture groups were found to be more toxic in kidney, thymus and adrenals compared to 100x and treatment with curcumin was effective and was observed more promising in 100x than 10x mixture. In toxicological studies, body weight, organ weight and relative organ weight, feed and water intake are considered to be important markers of toxicity (Monsour and Mosa, 2010; Mossa *et al.*, 2011).

Non-significant decrease in body weight but a significant reduction in percent weight gain, absolute and relative organ weight of testes and thymus are in correlation with the findings of different studies (Ibrahim *et al.,* 2012), binary metal mixture (Osfor *et al.,* 2010) and Jadhav *et al.* (2007) in a mixture of eight metals toxicity study. The dose-dependent decrease in feed and water intake was in agreement with Jadhav *et al.* (2007). The decrease in body weight, percent weight

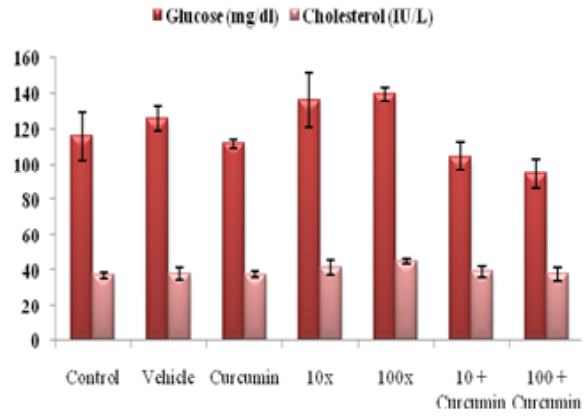

Fig. 1a

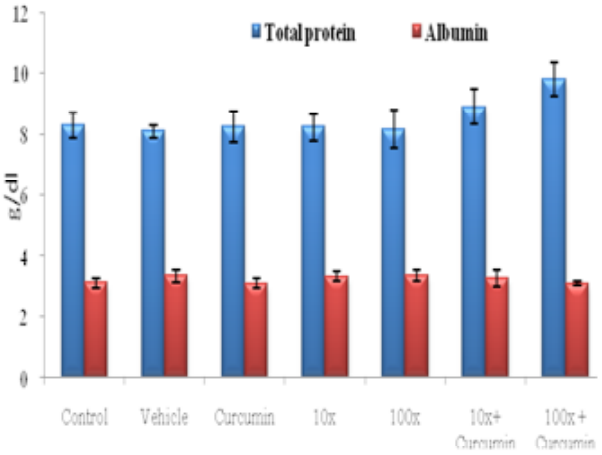

Fig. 1b

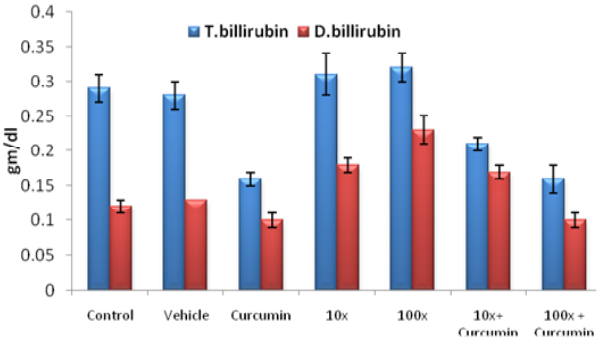

Fig. 1c

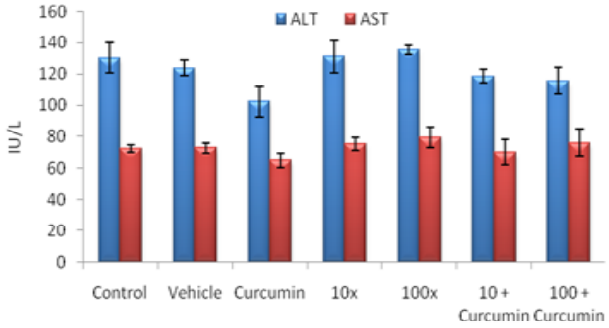

Fig. 1a-d:
Effects of metal mixture (10x and 100x), curcumin alone and in combination on blood glucose (mg/dl), total cholesterol (IU/L), total protein, albumin, total and direct bilirubin (g/dl) and enzymatic activities of ALT and AST (IU/L) following 28 days of continuous exposure.

gain, absolute and relative organ weight and feed and water intake may be caused by the toxic-ions associated with several factors, one of which is imbalance metabolism produced by impairing zinc status in zinc-dependent enzymes which are necessary for many metabolic processes (Ibrahim *et al.,* 2012). The progressive decrease in body weight at 100x exposure levels indicates growth retarding effect of the mixture as the duration of exposure advanced, which attributes to general systemic toxemia and degradation of lipids and protein as a result of mixture toxicity (Sidhu *et al.,* 2005). Reduction in feed and water intake in intoxicated groups in a progressive manner as the experiment advanced could be a contributing factor to weight loss and vice-versa and possibly the general metabolic condition of the animals would have not been with in the physiological limits (Jadhav *et al.,* 2007). This may be due to the adverse effects of the mixture on the tissues associated with biotransformation of the xenobiotics. The increase in the absolute weight of thymus and testes could be due to the magnification of the body's adaptive mechanisms to combat progression of toxicity.

The haematological parameters are presented in Tables 4, 5 and 6. The data reveals non-significant decrease in haemoglobin and total erythrocyte count in 10x and 100x exposure level while significant ($p<0.05$) decrease in packed cell volume and total leukocyte count was observed at 100x dose level. However the effect observed was dose dependent. Platelet count, mean corpuscular haemoglobin and hematocrit found to be increased in a dose–dependent manner, whereas insignificant alteration in mean corpuscular volume and mean corpuscular haemoglobin concentration was observed when compared to control and vehicle control groups. Concurrent treatment with curcumin, showed protective effect and improved the haematological parameters towards normal control values. Non-significant dose dependent decrease in hemoglobin, total erythrocyte count and packed cell volume, are in correlation with the previous reports (Boukerche *et al.*, 2007; Hounkaptin *et al.,* 2012), whereas significant increase in total leucocyte count was observed is 100x dose level. This finding is in agreeement with the Hounkaptin *et al.* (2012). The decrease in red blood cell

count, Hb and PCV may be due to the hemotoxic effects of these metals causing anaemia in wistar rats (Veena *et al,* 2011; Lavicoli *et al.,* 2003). Further the decreased Hb concentration in intoxicated rats is associated with the decrease in MCHC indicate a tendency to macrocytosis and hypochromic hematopoiesis in the liver which occurs efficiently. The decrease in total leukocyte count may be due to indication of leucopenia and thrombocytopenia in cases of severe liver dysfunction (Lee, 2004). The altered hematological parameters were correlated and an extent upto control values when the rats were treated with curcumin as dietary supplement. Curcumin has anti-oxidant potential and acts by preventing the binding of free radicals to the lipid membrane and further peroxidative damage to cells.

At the end of exposure period, 10x and 100x metals mixture groups showed significantly ($p<0.05$) increased plasma glucose level, non-significant increase in total protein, albumin, total and direct billirubin, total cholesterol (Figures 1a, 1b and 1c). Concurrent treatment with curcumin has been observed to be mild to moderately and significantly improve the biochemical indices in metals mixture exposed groups by reducing blood glucose, total cholesterol and protein content. The activities of ALT and AST were increased significantly in metal mixture exposed groups and significant reduction with curcumin treatment (Figure 1d). These findings are parallel with the previous metal toxicity studies (Jadhav *et al.,* 2007; Ibrahim *et al.,* 2012). The increase in the biochemical indices paralleled with the exposure level of metal mixtures indicate that the increment as dose-dependent. The hyperglycemic effect of 10x and 100x dose level might be due to vulnerable stress induced by the heavy metal mixture, as heavy metal toxicity increase the glucose content in blood due to intense glycogenolysis and increased synthesis of glucose from entero-hepatic tissue protein and amino acids (Almeida *et al.,* 2001). Non-significant but dose-dependent increase in blood cholesterol level was observed which can be an important risk factor for development of atherosclerosis. Liver is the primary organ for biotransformation of cholesterol and other endogenous substances. Increased cholesterol might be due to hepatic dysfunction as it is evident with increase in the level of total and direct bilirubin, AST and ALT which are indicators of increased liver microsomal membrane fluidity, free radical generation and hepato-cellular necrosis.

Evaluation of liver injury in toxicity trial is better judged by the battery of tests including abnormal levels of AST, ALT, bilirubin, total protein. Although most of these are not specific to the liver but if several of these are abnormal, then a hepatic cause is likely to be present (Pineiro canero and Pineiro, 2004). The common environmental metal pollutants, viz., lead, arsenic, cadmium and mercury are known to induce hepato-toxicity (Jadhav *et al.,* 2007; Ibrahim *et al.,* 2012). In the present study the metal mixture increased plasma activities of ALT and AST, decreased total plasma protein and increase in total and direct bilirubin, The present findings correlate with earlier studies (Brzoska *et al.,* 2003; Noriega *et al.,* 2003; Abdou *et al;* 2007; Jadhav *et al.,* 2007; Seddik *et al.,* 2010; Ibrahim *et al.,* 2012). Heavy metal-induced generation of free radicals and consequent oxidative stress is one of the important mechanisms that mediate death of hepatocytes (Valko *et al.,* 2005; 2006; Bashir *et al.,* 2006).

The present results showed that the effect of metal mixture on transaminase activity is dose-dependent. The elevation of plasma bilirubin may be due to induction of heme-oxygenase, which converts heme to bilirubin (Murrey *et al.,* 2006; Seddik *et al.,* 2010). Bilirubins also play an important protective role against oxidative damage of cell membrane-induced by metals (Noriega *et al.,* 2003, Zoheb *et al.,* 2014). Seddik *et al.* (2010) reported that heavy metals-induced heme-oxygenase is an enzyme that converts heme to bilirubin. Bilirubin formed in different tissues is transported to liver as a complex. Normally, bilirubin is conjugated with glucuronide in smooth endoplasmic reticulum of liver, but owing to peroxidation of lipid membrane of smooth endoplasmic reticulum, the conjugation of bilirubin with glucuronide may become inactive and bilirubin may exerts protective role against metal-induced oxidative damage. The reduction in total plasma protein with a simultaneous non-significant change in albumin is generally interpretated as a nonspecific indicator of toxicity and can be caused by several factors, including reduced feed intake, liver dysfunction, renal protein loss and inhibition of protein biosynthesis through specific enzymes of cell process (Jadhav *et al.* 2007; Ibrahim *et al.,* 2012).

From the present study, it is obvious that treating the mixture exposed rats with curcumin is beneficial in reducing and slowing down progressive damage which was accelerated by oxidative stress induced by metal mixture (Khan *et al.,* 2013; Zoheb *et al.,* 2014). Curcumin corrects antioxidant status by allowing different mehanisms. The antioxidant mechanism of curcumin is due to its specific conjugated structure of two methoxylated phenols and an enol form of β-diketone. This structure is responsible for the radical trapping as a chain breaking antioxidant (Masuda *et al.,* 2001). Also curcumin has the ability to chelates heavy metals (Daniel *et al.,* 2004) and significantly protects tissue insults against lipid peroxidation induced by heavy metals. Curcumin exhibits protective effects against oxidative damage by decreasing the level of free radicals,

through its free radical-scavenging activity, particularly against oxygen radicals, which inhibits –SH group oxidation (Manikandana *et al.,* 2004). The antioxidant mechanism of curcumin was attributed to its conjugated structure which includes two methoxylated phenols and an enol form of β-diketone. This structure showed a typical radical trapping ability as a chain breaking antioxidant (Masuda *et al.,* 2001).The preventive effect of curcumin is its ability to eliminate the hydroxyl radical (Reddy and Lokesh, 1994), superoxide radical (Sreejayan and Rao, 1996), single oxygen (Rao *et al.,* 1995), nitrogen dioxide (Unnikrishnan and Rao, 1995) and NO (Sreejayan and Rao, 1997).

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Research Article

STUDIES ON IMMUNOMODULATORY AND THERAPEUTIC EFFICACY OF A COMBINATION OF GARLIC, TURMERIC AND NEEM ON ENDOMETRITIS IN REPEAT BREEDING CROSSBRED COWS

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ABSTRACT

This study was designed to investigate the immunomodulatory and therapeutic efficacy of a combination of garlic, turmeric and neem on endometritis in repeat breeding crossbred cows. Twenty four repeat breeding crossbred cows were selected on the basis of history, breeding records, per rectal examinations of genitalia and white side test. All the animals were randomly divided into 3 groups viz; Group A: 30 ml normal saline; Group B: 30 ml Ciprofloxacin; Group C: 30 ml hydro-alcoholic extract of garlic, turmeric and neem. All the treatments were given intrauterine beginning on the day of estrus and continued for seven days in each group. Cervical mucus, blood and uterine flushing samples were collected at different intervals for appearance, pH, white side test and bacterial load, blood glucose, haemoglobin (Hb), packed cell volume (PCV), total leukocyte count (TLC),differential leukocyte count (DLC), total protein, immunoglobulin and polymorph nuclear cells (PMNs). At subsequent standing estrus following treatment, all cows were artificially inseminated twice using deep frozen semen at 12 hours interval. Pregnancy was confirmed perrectally on 45-60 days after insemination. Significant ($p<0.05$) decline in pH and bacterial load was observed in cervical mucus of all groups after treatment. Values of Hb, PCV, RBC, lymphocyte, nutrophils and glucose in blood samples were increased significantly ($p<0.05$) in group C. Similarly a significant ($p<0.05$) rise was found in TLC, PMN serum, total proteins and immunoglobulin concentration in uterine flushing samples of group C. Based on these results, it may be concluded that hydro-alcoholic extract of garlic, turmeric and neem combination has a good antibacterial and immunomodulatory property and can be used as a therapy for endometritis in repeat breeding crossbred cows.

Key Words- Repeat breeding, Endometritis, Herbs, Cervical mucus and uterine flushings.

INTRODUCTION

Repeat breeding with subclinical endometritis is a substantial problem in cattle breeding leading to huge economic loss to the dairy producers due to more inseminations, increased calving interval and culling rates (Lafi *et al.,* 1992). Several approaches have been employed to treat the endometritis including antibiotics, antiseptics and hormones, which have many side effects including drug resistance, disposal of milk and meat and high cost etc (Dhaliwal *et al.*, 2001). Herbs, being cheaper and safer than allopathic drugs, may be very useful in veterinary practice, especially in India, where they are found in plenty.

MATERIALS AND METHODS

Twenty four repeat breeding crossbred cows were selected in the Instructional Dairy Fram, Nagla, G.B.P.U.A.&T. Pantnagar on the basis of history, breeding records, per rectal examinations of genitalia and white side test. All the animals were randomly divided into 3 groups (Group A: 30 ml normal saline; Group B: 30 ml ciprofloxacin; Group C: 30 ml hydro-alcoholic extract of garlic, turmeric and neem. All the treatments were given intrauterine beginning on the day of estrus and continued for seven days in each group. Cervical mucus samples were collected on the day of estrus before treatment and at subsequent estrus after treatment and tested for appearance, pH, white side test and bacterial load. Blood samples and uterine flushings were also collected on the day of estrus before treatment and again on eight day of first collection i.e. 24 hr after last treatment. Blood samples were used to analyzed glucose, hemoglobin (Hb), packed cell volume (PCV), total leukocyte count (TLC) and differential leukocyte count (DLC) to know health status of the experimental animals while uterine flushings were used for the estimation of total protein, immunogloblobulin, TLC and polymorphonuclear cells (PMNs). At subsequent standing estrus following treatment, all cows were artificially inseminated twice using deep frozen semen at 12 hours interval. Pregnancy was confirmed perrectally on 45-60 days after insemination. The data were analyzed statistically using analysis of variance (ANOVA), paired *t*-test and chi-square test (Snedecor and Cochran, 1989).

RESULTS AND DISCUSSION

Clear estrual cervical mucus is conducive for sperm penetration and conception, whereas, turbidity retards sperm motility in estrual mucus (Dev *et al.*, 1997). Increase in pH may be due to metabolites of bacteria

Table 1:
pH (Mean±SE) of estrual cervical mucus in repeat breeding crossbred cows of different groups before and after treatment.

Groups	No. of animals	Before treatment	After treatment
Group A (Control)	8	7.77 ± 0.226^{Ba}	7.68 ± 0.161^{Aa}
Group B (Antibiotic)	8	7.81 ± 0.187^{a}	7.25 ± 0.945^{a}
Group C (Garlic+turmeric+neeml/U) Hydroalcoholic extract	8	7.87 ± 0.125^{Bb}	7.65 ± 0.188^{Aa}

Means bearing different superscripts vary significantly (p<0.05) within the groups (A, B) and between the groups (a, b,c).

Table 2:
Bacterial load (mean±SE) in estrual cervical mucus of repeat breeding cross bred cows of different groups before and after treatment (× 10^4/ml).

Groups	No. of animals	Before treatment	After treatment
Group A(Control)	8	301.29 ± 0.920^{Ba}	282.38 ± 1.030^{Aa}
Group B(Antibiotic)	8	308.58 ± 2.300^{Bb}	1.00 ± 0.039^{Abc}
Group H (Garlic+turmeric+neem) Hydroalcoholic extract	8	290.79 ± 2.29^{Bh}	1.25 ± 0.051^{Ac}

Means bearing different superscripts vary significantly (p<0.05) within the groups (A, B) and between the groups (a, b, c).

and inflammatory exudates in estrual cervical mucus (Salphale *et al.*, 1993). In the present study a significant decline in pH and bacterial load was observed in all groups after treatment (Table 1 and 2). This reduction in pH may be due to decline in bacterial load and inflammatory process in uterus after treatment (Shaktawat, 2005). This result showed that these herbs are antibacterial and immunomodulatory in nature and thus, reduced bacterial load and subsequently inflammation process (Owis *et al.*, 2005). After treatment, bacterial loads in Groups B and C were significantly lower ($p< 0.05$) as compared to control group A. The severe reduction in bacterial load after treatment herbal extract indicates its potent antibacterial action while the mild reduction in the control group could be attributed to natural uterine defense mechanisms.

The significant increase ($p< 0.05$) in the mean Hb, PCV, RBC, nutrophils and blood glucose values were observed in group Band C (Table 1). Significant increase in haemoglobin (Hb) was found in all treated groups indicating efficacy of treatment in improvement of general body condition. The importance of the Hb level has not been directly implicated in reproductive disorders, yet a decrease in Hb value is indicative of certain systemic disorders which could indirectly affect the functional activity of the reproductive organs. A low level of Hb influences tissue oxygenation of the reproductive tract, which in turn could affect the cyclicity (Ramakrishna, 1997). Thrall (2004) reported that in inflammatory disease, erythropoietin is diminished presumably because of inflammatory cytokines leading to lowered erythropoiesis and ultimately lowered PCV in blood. Lulay *et al.* (2011) found increased WBC numbers, especially neutrophils, in the estrous cycle and this effect is most likely due to the reproductive hormones dominating the cows plasma. Amin *et al.* (2010) also found that there was increase in RBCs after treatment with neem and turmeric. A significant increase ($p< 0.05$) in blood glucose was observed after treatment in all

Table 3.
Blood parameters of repeat breeding crossbred cows of different groups before and after treatment.

Parameter	Group A (Control)		Group B (Ciprofloxacin)		Group C Garlic+Turmeric+Neem (Hydroalcoholic extract)	
	Before treatment	After treatment	Before treatment	After treatment	Before treatment	After treatment
Hb (g/dl)	8.25±0.105	6.98 ± 0.121^{a}	9.85 ± 2.320^{A}	13.15 ± 0.379^{Bb}	8.62 ± 0.384^{A}	12.82 ± 0.568^{Bb}
PCV (%)	27.50±0.944	26.12 ± 0.81^{a}	25.00±2.32	34.12 ± 1.87^{b}	24.50 ± 0.566^{A}	34.87 ± 0.811^{Bb}
RBC (10^6/cumm)	4.72±0.243	4.00 ± 0.245^{a}	3.41±3.373	6.58 ± 0.263^{b}	3.65 ± 0.197^{A}	5.18 ± 0.085^{Bc}
Neutrophil (%)	43.75±2.366	37.62 ± 2.389^{a}	28.75 ± 0.940^{A}	39.87 ± 1.641^{Ba}	33.25±2.209	35.62 ± 2.129^{a}
Lymphocyte (%)	34.87±1.301	30.87 ± 1.619^{a}	36.62 ± 0.905^{A}	44.12 ± 0.914^{Bb}	46.75±4.813	49.00 ± 3.029^{b}
WBC (10^3/ml)	7.45 ± 0.225^{A}	6.78 ± 0.133^{Ba}	9.90±0.718	11.46 ± 0.618^{b}	11.16±1.409	9.27 ± 0.864^{c}
Glucose (mg/dl)	51.23 ± 0.545^{A}	53.25 ± 0.403^{Ba}	52.28 ± 0.853^{A}	71.93 ± 0.843^{Bb}	55.83 ± 2.82^{A}	68.37 ± 0.990^{Bc}

Different capital letters (A,B) indicate significant (P<0.05) difference between before and after treatment values within a group whereas, different small letters (a,b,c) indicate significant difference between groups in before treatment and after treatment values, respectively.

Table 4.
Uterine flushing parameters of repeat breeding crossbred cows of different groups before and after treatment .

Parameter	Group A (Control)		Group B (Ciprofloxacin)		Group C Garlic+Turmeric+Neem (Hydroalcoholic extract)	
	Before treatment	After treatment	Before treatment	After treatment	Before treatment	After treatment
TLC (10^6/ml)	0.422 ± 0.037^{A}	0.574 ± 0.024^{Ba}	0.618 ± 0.033^{B}	0.180 ± 0.031^{Ab}	0.591 ± 0.088^{A}	5.32 ± 0.201^{Bc}
PMN (%)	29.87 ± 0.440^{B}	27.12 ± 0.295^{Aa}	28.62 ± 0.625^{A}	20.12 ± 0.914^{Bb}	28.37 ± 0.497^{A}	61.00 ± 0.731^{Bc}
Protein (mg/dl)	159.99±3.518	164.34 ± 5.724^{a}	166.36 ± 1.396^{A}	137.29 ± 1.304^{Bb}	157.95 ± 0.730^{A}	180.24 ± 0.516^{Bc}
Total IG (mg/dl)	38.78 ± 0.584^{A}	42.05 ± 0.394^{Ba}	40.82 ± 0.263^{B}	32.70 ± 0.552^{Ab}	37.79 ± 1.195^{A}	59.00 ± 0.734^{Bc}

Different capital letters (A,B) indicate significant (P<0.05) difference between before and after treatment values within a group whereas, different small letters (a,b,c) indicate significant difference between groups in before treatment and after treatment values, respectively.

groups except control group. Mean blood glucose value in selected cows before treatment in the present study was lower than the value found in normal cyclic fertile cows. This finding is in agreement with Ramakrishna (1997).

Uterine flushing parameters (TLC, PMNs, total protein and total immunoglobulin) increased in group C after treatment with hydroalcoholic extract of turmeric+garlic+neem indicating its positive effect on the uterine defense mechanism. This increased intrauterine populations and oxidative burst activity of neutrophils favours the spontaneous resolution of uterine infection (Mateus *et al.*, 2002). Neutrophils are known to play a primary role in the defense of the uterus against infection. Influx of neutrophils into the uterus is thought to be mediated by chemoattractants, chemokines and adhesion molecules, such as β2-integrin and l-selectin (Tizard, 2000). Several workers have demonstrated a marked increase in protein concentration and PMNs infiltration in uterine flushings following uterine contamination (Strezemienski and Kenney, 1984). It is reasonable to assume that this response may play an important role in controlling bacterial infection introduced at coitus or parturition.

The cows were considered to have recovered from endometritis at subsequent estrus after treatment on the basis of clean appearance of estrus cervical mucus, reduction in bacterial load and negative white side test. The clinical recovery and conception rates in Groups A, B, and C were 25.0% and 0.0%, 87.5% and 62.5%, and 87.5% and 75%, respectively.

Thus, it may be concluded that hydro-alcoholic extract of turmeric, garlic and neem may be recommended for the treatment of infectious endometritis over conventional antibiotic treatments which have several demerits viz. uneconomical, drug resistance, milk withdrawal, residual effect and immunesuppression.

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Research Article

DISPOSITION OF ORALLY ADMINISTERED LEVOFLOXACIN IN LIPOPOLYSACCHARIDE INDUCED FEBRILE MODEL OF SHEEP

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ABSTRACT

The disposition of levofloxacin was evaluated following its single dose oral administration (3 mg kg^{-1}) in febrile sheep. The concentration of levofloxacin was measured using HPLC. The peak plasma concentration of 0.906 ± 0.043 μg/mL was observed at 4 h in febrile sheep. Following oral administration of the drug, the absorption ($t_{½Ka}$) and elimination half-lives ($t_{½\beta}$) were 0.89± 0.09 and 4.47± 0.23 h, respectively. The mean apparent volume of distribution ($Vd_{(area)}$), area under plasma drug concentration-time curve ($AUC_{(0-\infty)}$) and area under first moment curve (AUMC) were 2.63± 0.22 L/kg, 5.10± 0.23 μg.h/mL and 37.34± 1.24 μg.h^2/mL, respectively. The mean value of total body clearance (Cl_B) of the drug was 0.41 ± 0.03 L/h/kg with mean residence time (MRT) of 7.35± 0.20 h.Levofloxacin showed mediumoral bioavailability in febrile sheep and PK-PD relationship indicated that the drug may be efficacious against susceptible bacteria with MIC d" 0.06μg/mL.

Key words: Disposition, levofloxacin, oral administration, febrile condition, sheep.

INTRODUCTION

Antibacterial drugs are commonly used for the treatment of various bacterial infections in human and animals. In veterinary medicine, newer cephalosporinsand quinolones have been studied for their favorable pharmacokinetic profile (Tiwari *et al.,* 2009; Patel *et al.,* 2010; Patel *et al.,* 2012). Fluoroquinolones are rapidly absorbed following oral administration with moderate to good bioavailability in monogastric animals. Most of the newly developed fluoroquinolones are not significantly bound to plasma proteins. Therefore, free fluoroquinolone concentrations in serum generally reflect concentrations within the extracellular fluids, where the majority of infections occur. Overall, the newerfluoroquinolones display good penetration into alveolar macrophages, bronchial mucosa, epithelial lining fluid, and saliva. Pharmacokinetics of newer fluoroquinolones have been studied in animals and birds (Niteshkumar and Jaychandran, 2006; Patel *et al.,* 2009; Patel *et al.,* 2011).

Levofloxacin (LFX) is a third-generation fluoroquinolone that has been extensively used for the treatment of bacterial infections in human beings. It is active against Gram-negative organisms (including Pseudomonas species), Gram-positive organisms (including *Staphylococcus aureus*) and anaerobic bacteria. It has enhanced activity against *Streptococcus pneumoniae, Staphylococcus aureus* and Enterococcus species (Davis and Bryson, 1994). It has tremendous tissue penetration, large volume of distribution and relatively longer elimination half-life in animals. The drug thus seems to be extremely useful in a variety of infections including those of urinary tract, respiratory tract, soft tissues, bones and joints of animals. Pharmacokinetics and safety profile levofloxacin is extensively studied in animals (Patel *et al.,* 2009; Patel *et al.,* 2012; Patel *et al.,* 2013, Agarwal and Nitesh kumar, 2014) and birds (Patel *et al.,*2012; Varia *et al.,* 2009; Varia *et al.,* 2012; Patel *et al.,* 2019). Pharmacokinetic profile of levofloxacin following intravenous, subcutaneous and oral administration has been extensively studied with safety evaluation of the drug in sheep (Patel *et al.,* 2012; Patel *et al.*, 2013[b]; Patel *et al.,* 2019). The pharmacokinetics of levofloxacin in febrile goats have been studied by Mishra and Roy (2007) and they observed the alteration in pharmacokinetic profile of the drug. The disposition of levofloxacin duringfebrile condition in sheep following oral administrationhas not been studied yet. Thus, present study was carried out to evaluate the effect of febrile condition on disposition of levofloxacin following oral administration in sheep.

MATERIALS AND METHODS

Experimental animals

The experiment was carried out on six healthy female Patanwadi non-lactating sheep of 2-3 years old age obtained from and maintained at the Instructional Farm, College of Veterinary Science and Animal Husbandry, AAU, Anand, India. Constant observation for two weeks prior to commencement of the experiment was followed with clinical examination in order to exclude the possibility of any disease.The experimental protocol of the study was approved by the Animal Ethics

Committee.

Induction of febrile state

To study the disposition of levofloxacin during febrile condition in sheep, fever was induced by injecting lipopolysaccharide (LPS) of *Escherichia coli* (055:B5) at the dose rate of 0.2 µg/kg body weight intravenously (Verma, and Roy, 2006). This dose of lipopolysaccharide caused increase in temperature within 30 minutes, and fever persisted for 12 h. At least a 1.5 to 2.0°F increase of temperature from normal temperature was taken as the time of the drug administration (Agrawal *et al.,* 2002). Rectal temperature, respiratory rates and heart rates before and after administration of LPS in sheep under treatment were examined. LPS was again injected at dose rate of 0.1 µg/kg body weight at 12 h and at dose rate of 0.05 µg/kg body weight after 24 h of first dose of LPS to maintain the febrile state up to 36 h, respectively.

Drug administration and sampling

Levofloxacin oral tablet were procured from local pharmacy.Levofloxacin tablet (250 mg) was dissolved in 25 mL sterile water and used for oral administration using a syringe without the needle. Animals were fasted for 24 h before the oral administration of the drug. Blood samples (3 mL) were collected from i.v. catheter (Venflon, 22 × 0.9 × 25 mm) fixed into the right jugular vein into heparinized centrifuge tube. Following oral administration of the drug, blood samples were collected at 0 (prior to treatment), 0.083, 0.166, 0.33, 0.5, 0.75, 1, 2, 4, 6, 8, 12, 18, 24, 36 and 48 h post-treatment. Plasma was separated soon after collection by centrifugation at 3000 g for 15 min and transferred to labeled cryovials and stored at -35 °C until assayed for levofloxacin concentration using high performance liquid chromatography (HPLC) procedure.

Levofloxacin assay

Levofloxacin concentrations in the plasma samples were determined by HPLC with UV detection according to the method described previously(Patel *et al.*,2012[a]). The high performance liquid chromatography apparatus of Laballiance (USA) comprising quaternary gradient delivery pump (model AIS 2000) and UV detector (model 500) was used for assay. Chromatographic separation was performed by using reverse phase C_{18} column (Thermo, ODS; 250 × 4.6 mm ID) at room temperature. The HPLC data integration was performed using software Clarity (Version 2.4.0.190).

Pharmacokinetic and statistical analysis

Pharmacokinetic parameters were calculated using standard methods as described by Gibaldi and Perrier (1982).

PK/PD integration

Efficacy predictors like C_{max}/MIC_{90} and $AUC_{(0-\infty)}/MIC_{90}$ for concentration dependent antibiotic levofloxacin were calculated using the values of peak plasma drug concentration (C_{max}) and area under the curve ($AUC_{(0-\infty)}$) after oral administration in febrile sheep. There have been no studies reporting the MIC values of levoûoxacin from bacteria isolated from sheep. Therefore, in order to calculate the PK/PD efficacy predictors, hypothetical MIC values were used. An average plasma concentration of 0.032–0.2µg/ml has been reported to be the minimum therapeutic concentration (MIC_{90}) of levoûoxacin against most Gram-positive, Gram-negative and atypical bacteria (Chulavatnatol *et al.,* 1999). However, to cover most of the susceptible organisms, the MIC_{90} of 0.03, 0.06 and 0.1 µg/mL of levoûoxacin have been taken into consideration.

RESULTS AND DISCUSSION

Following oral administration of the levofloxacin, the drug concentration of 0.028 ±0.001 µg/mL was observed at 0.5 h. The mean peak plasma drug concentration of 0.906 ± 0.043 µg/mL was achieved at 4 h which declined rapidly to 0.103 ± 0.009 g/mL at 12 h. The drug concentration of 0.024 ± 0.002 µg/mL in plasma was detected at 24 h. The drug was not detected in samples collected after 24 h post oral administration of levofloxacin in febrile sheep.Levofloxacin concentration in plasma versus time following oral administration in febrile sheep is depicted as Figure 1. Pharmacokinetic parameters of levofloxacin following oral administration in febrile sheep are presented in Table 1. The mean absorption rate constant (K_a) and mean elimination rate constant (β) were 0.82± 0.08 and 0.16± 0.01 h^{-1}, respectively. The absorption ($t_{½Ka}$) and elimination half-lives ($t_{½\beta}$) were 0.89± 0.09 and 4.47± 0.23 h, respectively. The mean apparent volume of distribution ($Vd_{(area)}$), area under plasma drug concentration-time curve ($AUC_{(0-\infty)}$) and area under first moment curve (AUMC) were 2.63± 0.22 L/kg, 5.10± 0.23 g.h/mL and 37.34± 1.24 µg.h^2/mL, respectively. The mean value of total body clearance (Cl_B) of the drug was 0.41 ± 0.03 L/h/kg with mean residence

Table 1:
Pharmacokinetic parameters of levofloxacin after oral administration in febrile sheep

PK parameter	Unit	Values (Mean ± SE)
A'	µg/mL	0.82 ± 0.14
B	µg/mL	0.91 ± 0.12
K_a	h^{-1}	0.82 ± 0.08
β	h^{-1}	0.16 ± 0.01
$t_{½Ka}$	h	0.89 ± 0.09
$t_{½\beta}$	h	4.47 ± 0.23
C_{max}	µg/mL	0.91 ± 0.04
T_{max}	h	4.00 ± 0.00
$AUC_{(0-\infty)}$	µg.h/mL	5.10 ± 0.23
AUMC	µg.h^2/mL	37.34 ± 1.24
$Vd_{(area)}$	L/kg	2.63 ± 0.22
$Cl_{(B)}$	L/h/kg	0.41 ± 0.03
MRT	h	7.35 ± 0.20
MAT	h	5.40 ± 0.20

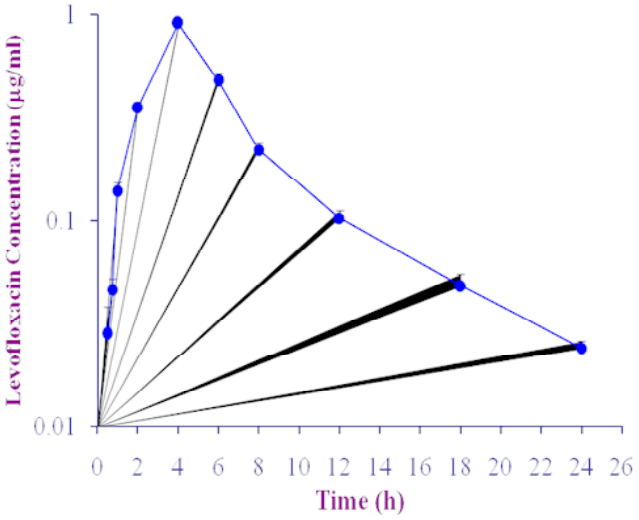

Fig 1:
Semilogarithmic plot of levofloxacin concentration in plasma versus time following oral administration in febrile sheep. Each point represents mean ± S.E of six animals.

time (MRT) of 7.35± 0.20 h. The calculated mean value of mean absorption time (MAT) was 5.40± 0.20 h. Following single dose oral administration of levofloxacin in healthy sheep, the absorption rate constant (Ka) and half-life ($t_{1/2ka}$) were reported to be 1.21 ± 0.19 h^{-1}, and 0.75 ± 0.23 h, respectively in study carried out by us previously (Patel *et al.,* 2019). The lower absorption rate constant and increased absorption half-life were seen in febrile sheep as compared to those in healthy sheep reported previously which clearly indicates slower absorption of the drug to some extent in febrile animals. The volume of distribution(Vd_{area}) of the drug in febrile sheep was lower than that (4.35 ± 0.29 L/kg) of healthy ones (Patel *et al.,* 2019). As fluoroquinolones have high lipid solubility and low plasma protein binding they are widely distributed in body and the same has been observed in the present study. In most species, the distribution volume of levofloxacin and other fluoroquinolones is greater than that for most β-lactam antibiotics and aminoglycosides, and probably represents intracellular sequestration of the drug in various tissues (Brown, 1996). Comparatively lower volume of distribution of the drug in febrile animals may be due to decrease in gastrointestinal and hepatic blood ûow in sheep given *E. coli* endotoxin. The decrease in the volume of distribution with fever could be related to alterations in the permeability of membranes and hemodynamic changes. This could contribute to reduce the volume of distribution of drugs including ûuoroquinolones, which have demonstrated a wide pass through ruminal epithelium (Gonzalez *et al.,* 2001).

The area under plasma concentration-time curve (AUC) and area under moment curve (AUMC) were found higher than those reported previously in healthy sheep (Patel *et al.,* 2019). Similar to our finding, the value of AUC was found higher in febrile goat following intravenous administration of levofloxacin (Mishra and Roy, 2007). Compared to reported total body clearance of the drug in healthy sheep (Patel *et al.,* 2019), the body clearance of the drug in febrile sheep was calculated to be lowered. Similar to our finding, the value of total body clearance of the levofloxacin was reported lower with increased MRT in febrile goat following intravenous administration (Mishra and Roy, 2007). The reasons of such variation in clearance of levofloxacin in sheep may be due to low protein binding, high lipid solubility and excretion as unchanged form, minimal tubular reabsorption and higher polarity of the drug(Fish and Chow, 1997). The elimination half-life ($t_{1/2\beta}$) after oral administration was longer than the elimination half-life following intravenous and subcutaneous administration of the drug in healthy sheep (Patel *et al.,*2012)indicates that drug is likely to act longer time after oral administration. Half-life depends on urinary pH of animal, and so may be different in various species of animals. It also depends on volume of distribution and clearance of the drug and in the present study, clearance was found lower. So, half-life was found to be high, as it is inversely proportional to clearance. We used data of our previous reports (Patel *et al.,* 2019) to calculate the bioavailability of the drug in the present experiment. The bioavailability of the drug in febrile sheep (63.29 ± 4.74 %) was higher than healthy sheep (Patel *et al.,* 2019). High bioavailability in the present study indicates moderate absorption via gastrointestinal tract after oral administration in febrile sheep. Moreover, therapeutically effective concentration produced and maintained for up to 24 hours suggest that oral administration of levofloxacin may be conventional for the management of bacterial infection caused by susceptible bacteria. The values of AUC/MIC and C_{max}/ MIC (85.00 and 15.17, respectively at MIC of 0.06µg/ mL)indicated that oral administration of levofloxacin in febrile sheep would be efficacious against bacteria with MIC of d" 0.06µg/mL. The values of AUC/MIC and C_{max}/ MIC in normal sheep reported to be 46.33 and 13.00 at MIC of 0.06 µg/mL in normal sheep(Patel *et al.,* 2019) which indicated the effect of febrile condition on pharmacokinetics and pharmacodynamics of the drug following oral administration in sheep.

It is concluded from this study that bioavailability of levofloxacin was medium after oral administration in febrile sheep. Based on PK-PD relationship calculation, it could be assumed that the drug might be efficacious against susceptible bacteria with MIC d" 0.06µg/mL.

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Research Article

MORIN POTENTIATES DUODENAL MOTILITY IN ARSENIC INTOXICATED SWISS ALBINO MICE

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ABSTRACT

The aim of the study was to study the effect of morin (a phytochemical) on duodenal motility in arsenic intoxicated mice. *Swiss albino* mice of 5-6 weeks of age were divided into three experimental groups containing six animals each such as; Gr-I (untreated and control), Gr-II (Arsenic trioxide @ 3 mg/kg bwt. p.o. for 28 days), Gr-III (morin @ 50 mg/kg bwt. p.o. + Arsenic trioxide @ 3 mg/kg bwt. p.o. for 28 days). Morin was administered 30 min before oral administration of arsenic trioxide. On 29th day the animals were sacrificed and a small piece of intestine from the duodenal part was collected in aerated chilled MKHS solution. 20 mm strips of the duodenum were mounted in organ bath containing 20 ml MKHS solution and given a resting tension of 0.5g and allowed one hour incubation period. After incubation period ACh (1nM-10uM) dose response curve was studied in viable rings. pD_2 of ACh in control was 6.95±0.18 which was decreased to 5.89±0.06 in arsenic intoxicated animals. This was elevated towards control value by administration of morin @50mg/kg (pD2= 7.04±0.22). EC_{50} in control (1.13×10^{-7}M) which was increased to 1.31×10^{-6} M in arsenic intoxicated group. EC_{50} was significantly decreased to 9.21×10^{-8}M which was very significant. In conclusion, from the functional study it was observed that morin at 50 mg/kg orally has potential effect on increasing duodenal motility and function in arsenic intoxicated swiss albino mice.

Key Words: Arsenicosis, Morin, Duodenum, As_2O_3

INTRODUCTION

Being one of the most toxic chemicals among the ten chemicals of major public health concern as per WHO guidelines, arsenic is a metalloid having both metallic and non-metallic properties naturally present at high levels in the ground water of a number of countries. Inorganic arsenic as an environmental contaminant is often found in water sources and has been found to elicit numerous toxic effects (Morzadec *et al.,* 2014). Exposure to inorganic arsenic has been linked to an increased incidence of gastroenteritis, cardiovascular disease, diabetes and various forms of cancer (Ramasamy and Lee 2015). Other evidence also suggests chronic exposure to arsenic causes liver injury, immune-toxicity, peripheral neuropathy and other neurotoxic effects. In populations chronically exposed to inorganic arsenic through drinking water, symptoms of dyspepsia, gastroenteritis and chronic diarrhoea have been reported (Guha Mazumder and Dasgupta, 2011). In rodents chronically exposed to high concentrations of monomethylarsonic acid [104 weeks; MMA(V) 10–1000/ mg/L], a metabolite of inorganic arsenic, it has been shown that the large intestine is the target organ (Arnold *et al.*, 2003), and there have been observations of enlargements of the intestinal wall, oedemas, haemorrhages and necrosis, ulcerations or perforations of the mucosa, as well as a significant increase in the incidence of squamous metaplasia of absorptive epithelial cells of the colon and rectum. *In vitro* studies have shown that acute exposure to trivalent forms of arsenic generates a pro-inflammatory response (increase of cytokines IL-6, IL-8, and TNFα) and produces oxidative stress (Calatayud *et al.*, 2014, Calatayud *et al.*, 2015) in human colonic epithelial cells.

All the data reported in the studies cited indicate that inorganic arsenic can have a toxic effect on a gastrointestinal level. The effects on the digestive system can affect important functions such as absorption or digestion of nutrients, and may also cause loss of the intestine's barrier function, which is necessary to avoid the passage of undesirable substances and microorganisms into the systemic circulation. Targeting metabolism by natural products is a novel approach and provides rationale for drug discovery. Therefore the aim of the present study is to study the effect of the flavonoid morin on duodenal motility in mice intoxicated with arsenic trioxide.

MATERIALS AND METHODS

Experimental animals

Swiss albino mice of 5-6 weeks of age were divided into three experimental groups containing six animals each such as; Gr-I (untreated and control), Gr-II (Arsenic trioxide @ 3 mg/kg bwt. p.o. for 28 days), Gr-III (morin @ 50 mg/kg bwt. p.o. + Arsenic trioxide @ 3 mg/kg bwt. p.o. for 28 days). Morin was administered 30 min before oral administration of arsenic trioxide. On 29th day the animals were sacrificed and a small piece

of intestine from the duodenal part was collected in aerated chilled MKHS (Modified Krebs Hanseliet Solution) solution. All experimental protocols were performed with due approval from Institutional Animal Ethical Committee (Approval No 06/IAEC/dt.09.03.18).

Isometric contraction study

20 mm strips of the duodenum were mounted in organ bath containing 20 ml MKHS solution and given a resting tension of 0.5g and allowed one hour incubation period. After incubation period ACh (1nM-10uM) dose response curve was studied in viable rings.

Data analysis and statistics

Contractile responses to ACh (1nM-10uM) on duodenal strips were expressed as the percentage of the spontaneous contraction response preceding acetylcholine (set at 100%). E_{max} and EC_{50} of ACh were determined by nonlinear regression analysis using Graph Pad Prism version 4 (San Diego, California, USA). Sensitivity/potency was expressed as pD_2= -log EC_{50}. Results were expressed as mean±SEM (n= number of animals). Data were analysed by two-way ANOVA for multiple comparisons followed by Dunet post-hoc test.

RESULTS AND DISCUSSION

The contractions of the circular and longitudinal muscles are regulated by electrical impulses that begin with the passage of calcium ions into the muscle cell. The duodenal pacemaker sends electrical impulses down the small intestine at a rate of 11 cycles per minute in the duodenum, gradually decreasing to 8 cycles per minute in the ileum. These electrical changes are propagated in the longitudinal muscle layer of the wall of the small intestine. The depolarization of the muscle cell membranes, or an excess of positive charges on the inside of the cell, causes the myofibrils (the contracting components of the myofilaments that constitute the muscle tissues) to contract. The rate of these contractions is governed by the rate of depolarization of the muscle cell membrane. The two spiral muscle layers then contract, causing the motor activity that permits the mixing and transporting of the food in the small intestine. A characteristic of small intestine motility is the inherent ability of the smooth muscle constituting the wall of the intestine to contract spontaneously and rhythmically. This phenomenon is independent of any extrinsic nerve supply to the small intestine. A peristaltic contraction may be defined as an advancing ring, or wave, of contraction that passes along a segment of the gastrointestinal tract. It normally occurs only over a short segment (approximately every 6 cm) and moves at a rate of about 1 or 2 cm per minute. This type of motor activity in the small intestine results in the transport of intraluminal contents downward, usually one segment at a time. When an inflammatory condition of the small bowel exists, or when irritating substances are present in the intraluminal contents, a peristaltic contraction may travel over a considerable distance of the small intestine; this is called the peristaltic rush. Diarrhoea due to common infections is frequently associated with peristaltic rushes.

Rice water diarrhoea is a characteristic symptom of arsenic poisoning. In populations chronically exposed to inorganic arsenic through drinking water, symptoms of dyspepsia, gastroenteritis and chronic diarrhoea have been reported. In rodents chronically exposed to high concentrations of monomethylarsonic acid there have been observations of enlargements of the intestinal wall, oedemas, haemorrhages and necrosis, ulcerations or perforations of the mucosa, as well as a significant increase in the incidence of squamous metaplasia of absorptive epithelial cells of the colon and rectum. In the present study we observed a peristaltic rush in the duodenal segment in the group of mice administered with arsenic trioxide p.o for 28 days [Fig 2] compared to the control animals [Fig 1] in agreement with earlier reports from other studies. Isometric contraction studies in duodenal segment revealed a peristaltic rush in duodenal motility disturbing

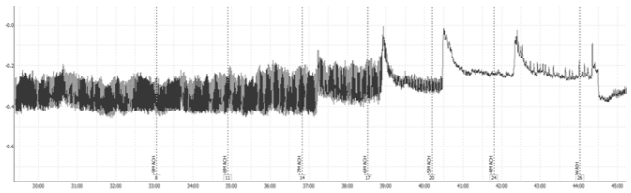

Fig. 1:
Gr I (Control)

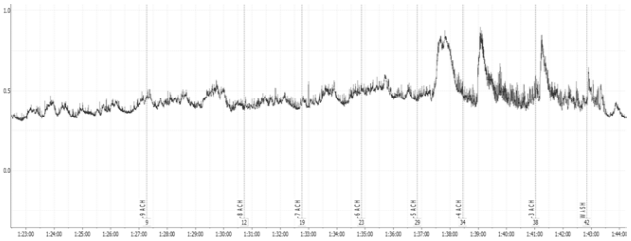

Fig. 2:
Gr II (Arsenic intoxicated)

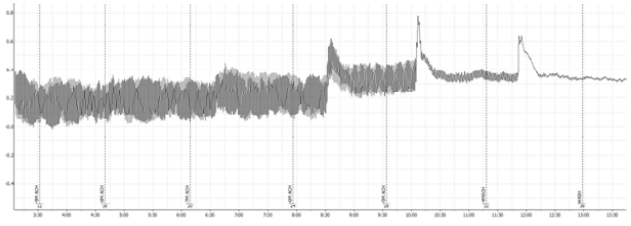

Fig. 3:
Gr III (treated with morin @50mg/kg p.o)

Table 1:
Effect of ACh (1nM – 10 µL) on duodenal motility in control (Gr I), morin @50mg/kg p.o (Gr III) and arsenic-intoxicated mice (Gr II) .

	Gr I	Gr II	Gr III
E_{max}	0.32±0.02g	0.41±0.01g	0.19±0.02g
pD_2	6.95±0.18	5.89±0.06	7.04±0.22
EC_{50}	1.13×10^{-7}M	$1.31^{*}\times10^{-6}$ M	$9.21^{**}\times10^{-8}$ M

[* p<0.05, **p<0.01 compared to Gr I (control), One-Way ANOVA, Dunet Test]

its rhythmicity and peak contractile response with response to ACh (1nM – 10 µL) [pD2: 5.89±0.06] compared to the control group [pD2: 6.95±0.18]. The rhythmicity and peak contractile response with response to ACh (1nM – 10 µL) was partially reversed towards control value by administration of morin @50mg/kg p.o (pD2= 7.04±0.22) [Fig 3]. The mean EC50 value in control group (1.13×10-7M) which was increased to 1.31×10-6 M in arsenic intoxicated group was found to decrease significantly to 9.21×10-8M (Table 1).

In conclusion, the present work suggests a possible effect of morin @50mg/kg p.o in potentiation of peristaltic rhythmicity of duodenum in mice intoxicated with arsenic trioxide. Nevertheless, further studies are required to elucidate the possible mechanisms.

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Research Article

DISPOSITION KINETICS AND DOSAGE REGIMEN OF CEFEPIME IN GOATS FOLLOWING SINGLE INTRAVENOUS ADMINISTRATION

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ABSTRACT

The disposition kinetics of cefepime after a single intravenous administration of 10 $mg.kg^{-1}$ was investigated in goats and an appropriate dosage regimen was calculated. The blood samples were collected at different time intervals and the concentration of drug in plasma was estimated by microbiological assay technique. At 1 min after injection, the concentration of cefepime in the plasma was 60.6 ± 0.70 $\mu g.ml^{-1}$, which declined to 0.23 ± 0.01 $\mu g.ml^{-1}$ at 24 h. The plasma concentration time data of cefepime was best fitted to two-compartment open model. The cefepime was rapidly distributed from the blood to the tissue compartment as shown by the high values for the initial distribution rate constant (8.10 ± 0.24 h^{-1}) and the constant for the rate of transfer of drug from central to the peripheral compartment, K_{12} (5.20 ± 0.12 h^{-1}). The elimination half-life and volume of distribution were 3.00 ± 0.05 h and 0.52 ± 0.02 $L.Kg^{-1}$, respectively. The total body clearance (Cl_B) and the ratio of the drug present in the peripheral to that in the central compartment (T/P ratio) were 0.118 ± 0.01 $L.Kg^{-1}.h^{-1}$ and 2.53 ± 0.11, respectively. On the basis of the pharmacokinetic parameters obtained in the present study, an appropriate intravenous dosage regimen of cefepime in goats for most of the bacteria sensitive to it would be 8.46 $mg.kg^{-1}$ repeated at 12 h intervals.

Key words: Goats, cefepime, dosage, pharmacokinetics.

INTRODUCTION

Cefepime, a recently introduced cephalosporin is being frequently employed for empirical therapy in severely ill patients in intensive care, oncology and transplantation units (Rule *et al.,* 1996). It has potent bactericidal activity against a broad range of gram-negative and gram-positive organisms including *Pseudomonas aeruginosa* and methicillin susceptible *Staphylococcus* spp. It is also highly active against many members of the family *Enterobacteriaceae* (Bertram *et al.,* 1984). If a drug is to be used effectively, it is important to investigate its pharmacokinetics in each animal species and climate in which the drug is to be used clinically (Nawaz *et al.,* 1980). The disposition kinetics of cefepime has been investigated in cow calves (Pawar and Sharma, 2007), monkeys (forgue *et al.,* 1987), rats (Brindley *et al.,* 1991), dogs (Gardner and Papich, 2001), neonatal foals (Gardner and Papich, 2001), ewes (Ismail, 2005a) and buffalo calves (Jain, 2009). However, there is little information available on the pharmacokinetics of cefepime in goats. In view of the marked species variations in the kinetic data of antimicrobial drugs, the present study was undertaken to determine the disposition kinetics and an appropriate dosage regimen for cefepime in goats after a single intravenous administration.

MATERIALS AND MATHODS

The experiment was performed on 6 healthy female goats, 2-2.5 years old and weighing 25-30 kg. The animals were adapted to laboratory conditions for 3 weeks prior to the commencement of the study and were provided dry as well as green fodder, concentrates, a routine grazing for at least 4-5 hours a day and water *ad libitum*. The average day temperature in the shed was about 25°C during the experimental period. The experimental protocol followed the ethical guidelines on the proper care and use of animals. Cefepime (Sefdin: Unichem Laboratories, Mumbai, India) was administered at a dose rate of 10 $mg.kg^{-1}$ body weight into the left jugular vein. Blood samples were withdrawn from the contralateral jugular vein into heparinized glass centrifuge tubes at 1, 2.5, 5, 7.5, 10, 15, 30, 60 min and 2, 4, 6, 8, 10, 12, and 24 after administration of drug. Plasma was separated by centrifugation at 3000 rpm for 15 min at room temperature and stored at –20 °C until analysis, which usually took place on the day after collection.

The concentration of cefepime in the plasma samples were determined by a standard microbiological bioassay technique (Arret *et al.,* 1971) using *Escherichia coli* (MTCC 739) as the test organism. The test organisms were cultured on medium No. 1 at 37°C for 24 h and a suspension was prepared in sterile normal saline. Assay plates were prepared by putting 25 ml of seed layer of medium No. 11 poured on the flat bottom of 100 ml of capacity assay petridishes. A desired amount of bacterial suspension was added to seed layer to obtain clarity of bacterial growth and the required dimensions of zone of inhibition with a reference concentration (1.00 $\mu g.ml^{-1}$) of cefepime. Preliminary

experiments were conducted to determine the actual amount of bacterial suspension to be used in the preparation of seed layer. After solidification of the media, six wells were punched at equal distances with the help of a punching machine (designed and standardized in our laboratory). The alternate three cylindrical wells were filled with one plasma sample and the remaining three cylindrical wells with a standard solution of cefepime (1.00 μg.ml^{-1}). These assay plates were incubated at 32°C for 6 h. At the end of incubation, the diameter of zone of inhibition of each well was measured.

Pharmacokinetic parameters were calculated manually by the computed least-square linear regression technique (Gibaldi and Perrier, 1982). Different estimates of the volume of distribution were obtained from the following equation:

$$Vd_{area} = \frac{\text{Dose (mg.kg}^{-1})}{\beta . AUC}$$

$$Vd_{B} = \frac{\text{Dose (mg.kg}^{-1})}{B}$$

$$Vd_{SS} = \frac{\text{Dose (mg.kg}^{-1}) . AUMC}{AUC^2}$$

The dose of cefepime (D – Priming Dose and D/ - Maintenance Dose), at various dosage intervals, for maintaining different MIC's was calculated from

$D = Cp (min)^{\propto} . Vd_{area} (e^{\beta\hat{o}})$

$D/ = Cp (min)^{\propto} . Vd_{area} . (e^{\beta\hat{o}} – 1)$

Where β is the elimination rate constant and ô is the dosage interval.

RESULTS

The plasma levels of cefepime at different time intervals are presented in Figure 1. The plasma concentration of cefepime at 1 min after a single intravenous injection was 60.6 ± 0.70 μg.ml^{-1}, which declined rapidly to 12.9 ± 0.62 μg.ml^{-1} at 1 h. The drug was detected in plasma for upto 24 h after dosing (0.23 ± 0.01 μg.ml^{-1}). The pharmacokinetic parameters that described the distribution and elimination pattern of cefepime were calculated and are presented in Table 1.

DISCUSSION

Intravenous administration of an antibiotic is the most reliable means of managing very ill patients and severe bacterial infections as initially very high antibiotic concentration in central compartment is desired to obtain favourable diffusion gradient from blood to tissues. To characterize pharmacokinetic pattern of cefepime in healthy goats, cefepime was administered by single intravenous injection at the dose rate of 10 mg.kg^{-1} body weight. The dose of cefepime used in the study is comparable to the dosage used in monkeys (Forgue *et al.*, 1987), rats (Brindley *et al.*, 1991), dogs (Stampley *et al.*, 1992), horses (Guglick *et al.*, 1998), neonatal foals (Gardner and Papich, 2001), goats (Patani *et al.*, 2006), calves (Pawar and Sharma, 2008a), buffalo calves (Jain, 2009), and humans (Barbhaiya *et al.*, 1990). Injection of antimicrobials directly into blood stream produces immediately high plasma concentration, thus in severe infections, i.v. administration of drug is required.

The plasma concentrations of cefepime following its i.v. injection were determined at different time intervals and a semi logarithmic plot of mean plasma concentration versus time was drawn. The evaluation of results indicated that disposition of cefepime in plasma after i.v. administration fitted a two-compartment open pharmacokinetic model and the pharmacokinetics was described by the following equation:

$$C_p = Ae^{-\alpha t} + Be^{-\beta t}$$

Where, C_p is the plasma concentration at time t, A and B are the zero time plasma concentration intercepts of biphasic disposition curve, α and β are hybrid rate constants related to the slope of the distribution and elimination, respectively and e is the base of natural logarithm.

Cefepime after i.v. administration has also been reported to follow two-compartment open model in dogs (Nakanomyo *et al.*, 1992), humans (Barbhaiya *et al.*, 1990), horses (Guglick *et al.*, 1998), foals (Gardner and Papich, 2001), ewes (Ismail, 2005b), calves (Patel *et al.*, 2006; Pawar and Sharma, 2008a), goats (Patani *et al.*, 2006) and buffalo calves (Jain, 2009).

The highest plasma concentration of cefepime (60.6 ± 0.70 μg.ml^{-1}) was obtained at 1 min. The concentration declined rapidly to 12.9 ± 0.62 μg.ml^{-1} at 1 h and after that cefepime gradually disappeared from plasma and a concentration of 0.23 ± 0.01 μg.ml^{-1} was detected at 24 h. The peak plasma level was approximately 60 fold higher than minimum therapeutic level of cefepime (1 μg.ml^{-1}) and the drug was detected above MIC upto 12 h. In accordance to the present findings, almost similar value of C_{max} 55.3 ± 0.54 μg.ml^{-1} in plasma was achieved after i.v. administration of cefepime at the dose rate of 10 mg.kg^{-1} b.wt. and the therapeutic concentration of cefepime was maintained in serum up to 12 h in cross-bred calves (Pawar and Sharma, 2008a). The present result is in contrast to the low value of $C_{max,}$ 44.9 ± 5.47 μg.ml^{-1} at 2 min in Holstein-Friesian calves (Patel *et al.*, 2006), 46.4 ± 0.40 μg.ml^{-1} at 1 min in buffalo calves (Jain, 2009). Similarly,

Nakanomyo *et al.* (1992) detected 83.5 μg.ml^{-1} of cefepime at 5 min after its single i.v. dose of 20 mg.kg^{-1} b.wt. in dogs.

For cefepime, the minimum therapeutic plasma concentration against majority of gram-positive and gram-negative pathogens in animals has been reported to be 0.008 – 1.5 μg.ml^{-1} (Knudsen *et al.*, 1997; Mimoz *et al.*, 1997; Miyazaki *et al.*, 2004; Pawar and Sharma, 2008a). Keeping in view the influence of certain unavoidable factor *in vivo*, in this discussion, the MIC of 1 μg.ml^{-1} of cefepime has been taken into consideration.

The low value of distribution half-life of cefepime (0.33 ± 0.13 h) indicated the rapid distribution of drug from central to peripheral compartment in healthy goats. The value of $t_{1/2\alpha}$ obtained in the present study was close to the value of 0.30 h in foals (Gardner and Papich, 2001), 0.36 h in horses (Guglick *et al.*, 1998) and 0.39 h in dogs (Nakanomyo *et al.*, 1992). However, the present result is in contrast to the low values of $t_{1/2\alpha,}$ 0.07 h in buffalo calves (Jain, 2009), 0.09 h in Holstein-Friesian calves (Patel *et al.*, 2006) and 0.20 h in cow calves (Ismail, 2005a). The rapid distribution of cefepime was further confirmed by the high values of K_{12}/K_{21} ratio (2.28 ± 0.9).

The volume of distribution (Vd) relates the amount of drug in the body to the concentration of drug in plasma. It refers to fluid volume that would be required to contain all the drug in the body at the same concentration as in the blood or plasma (Benet *et al.*, 1996). The volume of distribution varies widely depending on degree of plasma protein binding, pKa of drug and binding to the tissues. In the present study, the value of Vd_{area} (0.52 ± 0.02 L.kg^{-1}) in goats indicated moderate distribution of drug into various body fluids and tissues. In agreement to the present findings, similar value of Vd_{area} has been reported in Surti goats (0.52 L.kg^{-1}) after single i.v. administration of cefepime (Patani *et al.*, 2006). Almost similar value of Vd_{area} (0.57 L.kg^{-1}) has also been reported in Holstein-Friesian calves by Patel *et al.* (2006) and in buffalo calves by Jain (2009). However, the present results are in contrast to the low values of Vd_{area}, 0.225 L.kg^{-1} in horses (Guglick *et al.,* 1998), 0.19 L.kg^{-1} in cross-bred calves (Pawar and Sharma, 2008a) and 0.33 L.kg^{-1} in buffalo calves (Joshi, 2005). The moderate value of Vd_{area} in goats suggested that cefepime is distributed principally through extracellular fluid space. Despite low values of Vd_{area} for cefepime in most species, efficacy against infections located in various restricted compartments, such as CNS, has been documented (Grassi and Grassi, 1993). Apparently, the low degree of plasma protein binding and probable decrease in integrity of blood-brain barrier caused by inflammation promotes attainment of therapeutic concentrations of cefepime in brain tissues (Guglick *et al.*, 1998).

The mean value of volume of distribution at steady state (Vd_{ss}) was higher (0.49 ± 0.01 L.kg^{-1}) than the values of 0.46 L.kg^{-1} in rats (Forgue *et al.,* 1987), 0.43 L.kg^{-1} in calves (Patel *et al.*, 2006) 0.35 L.kg^{-1} in goats (Patani *et al.*, 2006), 0.32 L.kg^{-1} in ewes (Ismail, 2005b), 0.254 L.kg^{-1} in monkeys (Forgue *et al.*, 1987), 0.14 L.kg^{-1} in dogs, 0.18 L.kg^{-1} in foals (Gardner and Papich, 2001) and 0.24 L.kg^{-1} in humans (Barbhaiya *et al.*, 1992), indicating good extravascular distribution of drug in goats. As with other cephalosporins, the extravascular distribution of cefepime was limited to the extracellular fluid (Balant *et al.*, 1985). In spite of its limited distribution into the extracellular fluid as reported in most species studied, it appears that low protein binding and high

Table 1.
Disposition kinetic parameters of cefepime in goats following a single intravenous dose of 10 mg.kg^{-1} body weight

Parameter	Unit	Animal number						Mean ± SE
		1	2	3	4	5	6	
C_p^o	μg.ml^{-1}	67.0	64.3	68.6	65.4	69.5	68.9	67.3 ± 0.88
$t_{½\alpha}$	h	0.08	0.09	0.08	0.09	0.80	0.82	0.33 ± 0.13
$t_{½\beta}$	h	3.01	3.15	2.89	3.15	2.89	2.89	3.00 ± 0.05
K_{12}/K_{21}	ratio	2.22	2.38	1.98	2.61	2.29	2.19	2.28 ± 0.90
AUC	μg.ml^{-1}.h	83.9	80.0	90.3	75.1	81.9	83.6	82.5 ± 2.27
AUMC	μg.ml^{-1}.h^2	339.0	335.7	353.4	311.1	317.0	324.3	330.1 ± 7.01
Vd_{area}	L.kg^{-1}	0.52	0.57	0.46	0.60	0.53	0.51	0.52 ± 0.02
Vd_B	L.kg^{-1}	0.56	0.62	0.49	0.67	0.55	0.54	0.57 ± 0.02
Vd_{ss}	L.kg^{-1}	0.48	0.52	0.43	0.55	0.47	0.47	0.49 ± 0.01
Cl_B	L.kg^{-1}.h^{-1}	0.11	0.12	0.11	0.13	0.12	0.12	0.118 ± 0.01
K_{el}	h^{-1}	0.80	0.80	0.76	0.87	0.84	0.82	0.81 ± 0.01
MRT	h	4.03	4.21	3.91	4.14	3.86	3.88	4.00 ± 0.05
T/P	ratio	2.47	2.65	2.17	2.96	2.53	2.43	2.53 ± 0.11
td	h	10.0	10.5	9.58	10.4	9.57	9.58	9.94 ± 0.17

Cp^0, plasma drug concentration at zero time; $t_{1/2\alpha}$ and $t_{1/2\beta}$, half-lives of distribution and elimination phases, respectively; K_{12} and K_{21}, rate constants defined in the two compartment model; AUC, area under the plasma concentration-time-curve; AUMC, area under the first moment of the plasma concentration-time-curve; Vd_{area}, Vd_B and Vd_{ss} volume of distribution from AUC, elimination phase and steady state plasma level, respectively; Cl_B, total body clearance of the drug; K_{el}, elimination rate constant from the central compartment; MRT, mean residence time of drug in body; T/P, ratio of the drug present in the peripheral to central compartment; td, total duration of pharmacological effect.

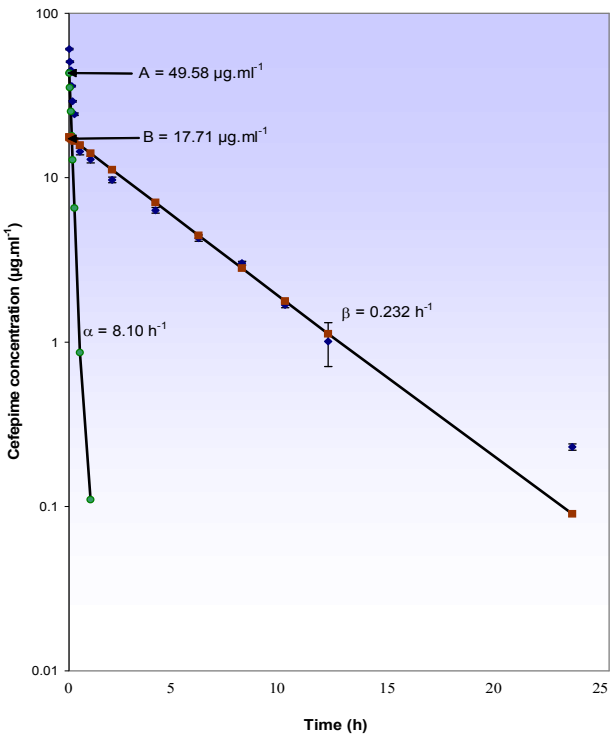

Figure 1: Semilogarithmic plot of plasma concentration-time profile of cefepime in healthy goats following a single intravenous dose of 10 mg.kg^{-1} body weight.

- Data was analyzed according to two compartment open model. the distribution (α) and elimination (β) phases are represented by least square regression lines. The calculated points (ï) of the distribution phase were obtained by feathering technique.
- Values denote Mean ± S.E. of six animals.

bactericidal activity are considered the major determinants for promotion of its therapeutic efficacy in all species (Kalman *et al.*, 1992). To further confirm the distribution pattern of cefepime in various body fluids and tissues of goats, the cefepime levels in central and peripheral compartments were computed and drawn on a semi logarithmic scale. It is reported that such diagrammatic representation of disposition of drug gives a guideline to evaluate the distribution pattern of drug in general, however, if the drug truly follows two-compartment open model and injection made rapidly by i.v. route, the calculated drug level in central and tissue compartments at various time intervals may be used as an excellent index to determine the rate and extent of distribution of drug from central to peripheral compartment. The pattern of movement of cefepime from central to peripheral compartment suggested that the built up of cefepime concentration in tissue compartment was very slow. While comparing the figure with the calculated data, it is revealed that the figure is the exact mirror image of the distribution pattern of cefepime. Further the T/P ratio of 2.53 ± 0.11 observed in the present study, reflected high concentration of cefepime in body fluids and tissues as compared to that in plasma of goats.

The high value of AUC (82.46 ± 2.27 µg.ml^{-1}.h) and AUMC (330.08 ± 7.01 µg.ml^{-1}.h^2) reflected that vast body area is covered by drug concentration in goats. Similarly, high value of AUC following single i.v. injection have been reported for cefepime as 70.2 ± 0.52 µg.ml^{-1}.h in buffalo calves (Jain, 2009), 78.4 µg.ml^{-1}.h in goats (Patani *et al.*, 2006), 94.5 µg.ml^{-1}.h in cow calves (Ismail, 2005a), 114.8 µg. ml^{-1}.h in dogs and 182.5 µg.ml^{-1}.h in foals (Gardner and Papich, 2001). In contrast to present findings, low value of AUC (47.7 µg.ml^{-1}.h) was reported in calves by Patel *et al.* (2006).

The elimination half-life ($t_{1/2\beta}$) is the time taken for plasma concentration in the body to be reduced by 50 per cent. Half-life provides a good indicator of time required to reach steady state after a dosage regimen has been initiated. The elimination half-life of cefepime in goats in the present study was 3.00 ± 0.05 h. In accordance to the present findings, almost similar values of $t_{1/2\beta}$, 2.75 h in buffalo calves (Jain, 2009), 2.71 h in goats (Patani *et al.*, 2006) and 2.67 h in calves (Joshi, 2005) were observed after single i.v. administration of cefepime. In contrast, cefepime was rapidly eliminated in cross-bred calves, ewes, foals and dogs. The value of $t_{1/2\beta}$ has been reported as 1.28 h in cross-bred calves (Pawar and Sharma, 2008a), 1.76 h in ewes (Ismail, 2005b), 1.65 h in foals and 1.09 h in dogs (Gardner and Papich, 2001). The elimination rate constant of cefepime from central compartment (K_{el}) was 0.81 ± 0.01 h^{-1}, which was almost similar to the value of 0.79 h^{-1} in dogs (Gardner and Papich, 2001). However, comparatively lower value of K_{el}, 0.29 h^{-1} in cow calves (Ismail, 2005a) and 0.36 h^{-1} in horses (Guglick *et al.*, 1998) was observed.

Total body clearance (Cl_B) of cefepime, which represents the sum of metabolic and excretory processes was 0.118 ± 0.01 L.kg^{-1}.h^{-1} in goats. The value of total body clearance in the present study was comparable to the value reported as 1.18 ml.min^{-1}.kg^{-1} in horses (Guglick *et al.*, 1998) and 126 ml.kg^{-1}.h^{-1} in human (Garrelt and Wagner, 1999) but slightly lower than the values reported as 0.139 L.kg^{-1}.h^{-1} in buffalo calves (Jain, 2009) and 1.33 ml.min^{-1}.kg^{-1} in foals (Gardner and Papich, 2001), respectively, following i.v. administration of cefepime.

The value of mean residence time (MRT) of cefepime in goats (4.00 ± 0.05 h) was almost similar to the value reported as 3.38 h in cow calves (Ismail, 2005a), 3.5 h in buffalo calves (Joshi, 2005), 3.77 ± 0.06 h in buffalo calves (Jain, 2009) and 3.95 h in Holstein-Friesian calves (Patel *et al.*, 2006), but longer than the values of 2.03, 2.16 and 1.05 h reported in horses (Guglick *et al.*,

1998), foals and dogs (Gardner and Papich, 2001), respectively.

The main objective of this pharmacokinetic study was to compute the most appropriate dosage regimen of cefepime in goats. The most appropriate priming and maintenance doses of cefepime to maintain an MIC of 1.00 $\mu g.ml^{-1}$ in goats, at a dosage interval of 12 h would be 8.46 and 7.92 $mg.kg^{-1}$, respectively. Similarly, cefepime dosage regimen in calves is reported to be 8.3 $mg.kg^{-1}$ b.wt. at 12 h intervals (Joshi, 2005). In contrast to the present results, dosage regimen of cefepime recommended in horses, Holstein-Friesian calves, buffalo calves, cross-bred calves and dogs were 2.2 $mg.kg^{-1}$ at 8 h intervals (Guglick *et al.*, 1998), 4.20 $mg.kg^{-1}$ at 12 h intervals (Patel *et al.,* 2006), 11.5 $mg.kg^{-1}$ at 12 h intervals (Jain, 2009), 15.5 $mg.kg^{-1}$ at 8 h intervals (Pawar and Sharma, 2008a) and 40 $mg.kg^{-1}$ at 8 h intervals (Gardner and Papich, 2001), respectively.

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Research Article

IN VITRO ANTIOXIDATIVE ACTIVITY OF *WITHANIA SOMNIFERA* AQUEOUS ROOT EXTRACT

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ABSTRACT

The present study was undertaken to evaluate the antioxidant and radical scavenging potentials of *Withania somnifera* aqueous root extract (WRE) by 3 different assays: DPPH Free radical scavenging assay, Fe^{2+} chelating assay and reducing power assay. The results showed that DPPH scavenging activity increased with increase in concentration of WRE when compared to standard antioxidant, butylated hydroxytoulene (BHT). Reducing power of WRE was significantly less when compared to BHT but increased dose dependently with maximum activity at highest concentration (1 mg/ml). The aqueous extract of WRE disrupted ferrous-ferrozine complex and showed increase in chelating activity in a dose dependent manner. WRE contains phenolic compounds and glycowithanolides (Sitoindosides VII-X and Withaferin A) which donates electron and hydrogen to protect against free radical mediated autoxidation of unsaturated lipids. It is, therefore, inferred that WRE possess *in vitro* antioxidant activity.

Key words: *Withania somnifera*, *in vitro* antioxidative activity etc.

INTRODUCTION

The use of *Withania somnifera* as antistress, antioxidative, antiaging, anti-inflammatory and rejuvenating substance is documented in ayurvedic and Middle Eastern traditional medicines (Mishra *et al.*, 2000). Due to antioxidative action of *Withania somnifera's* active constituents - Sitoindoside VII and Withaferin A, clinical trails and animal researches support its use in cognitive, neurological disorders, inflammation and Parkinson's disease (Bhattacharya *et al.*, 1997, Panda and Kar, 1998, Sunanda *et al.*, 1998). The present investigation was undertaken to evaluate the antioxidative activity of *Withania somnifera in vitro.*

MATERIALS AND METHODS

Withania somnifera root powder was soaked in water overnight, filtered and filterate was dried and lypolized to make *Withania somnifera* aqueous root extract (WRE). Antioxidative potential of the extract (WRE) was evaluated in terms of 2, 2'-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging ability, effect on the chelating activity of Fe^{2+} and the reducing power in comparison to the synthetic antioxidants. Butylated hydroxytoluene (BHT) was taken as synthetic antioxidant. All determinations were performed in triplicate.

Reducing power activity

The reducing power of WRE was determined by the methods reported earlier (Yen and Duh, 1993 and Singh *et al.*, 2005). Zero point five microliter of various concentrations of WRE (0.1, 0.25, 0.5, 0.75, 1 mg/ml) was mixed with 2.5 ml of the phosphate buffer (200 mM, pH 6.6) and 2.5 ml of 1% potassium ferricyanide. The mixtures were incubated at 50°C for 20 min. After incubation, 2.5 ml of 10% trichloroacetic acid was added to the mixtures, followed by centrifugation at 650 g for 10 min. The upper layer (5 ml) was mixed with 5 ml of distilled water and 1 ml of 0.1% ferric chloride and the absorbance of the resultant solution were measured at 700 nm using UV Vis-spectrophotometer.

Same procedure was applied to control (without extract) and BHT standards (0.1, 0.25, 0.5, 0.75, 1 mg/ml) which served as negative and positive controls, respectively. Absorbance at 700 nm is plotted against the different concentrations of WRE. Increase in reducing power was indicated by increase in absorbance.

Chelating activity of WRE on Fe^{2+}

The chelating activity of WRE on ferrous ions (Fe^{2+}) was measured by the method reported by Juntachote and Berghofer (2005). One microliter different concentrations of WRE (0.1, 0.25, 0.5, 0.75, and 1.0 mg/ml) were mixed with 3.7 ml of deionised water. The mixtures were left for reaction with $FeCl_2$ (2 mM, 0.1ml) and ferrozine (5 mM, 0.2 ml) for 10 min at room temperature and then absorbance was measured at 562 nm using UV Vis-spectrophotometer. A lower absorbance indicates the higher chelating power. The chelating activity on Fe^{2+} of WRE was compared with that of EDTA at a level of 0.01 mM and citric acid at a level of 0.025 M. Chelating activity was calculated by the following formula

$$\text{Chelating activity (\%)} = [1 - (A_t / A_o)] \times 100$$

(A_t is the absorbance of the sample at 562 nm and A_o is the absorbance of the control at 562 nm).

DPPH radical scavenging activity

The scavenging effect of WRE on the DPPH radical was determined according to the methods developed earlier (Yen and Duh, 1993; Cuendet *et al.*, 1997 and Singh *et al.*, 2005). Zero point one microliter of different concentrations of WRE (0.1, 0.25, 0.5, 0.75, 1 mg/ml) were mixed with 5 ml of 0.004% methanolic solution of DPPH. Each mixture was incubated for 30 - 45 min in the dark and the absorbance of the sample was read at 515 nm using UV Vis-spectrophotometer. The DPPH solution was freshly prepared daily, stored in a flask, covered and kept in dark at 4°C between the measurements. The control and standard samples were subjected to the same procedure except for the control, where there was no addition of the sample and for the standard 0.1, 0.25, 0.5, 0.75, 1 mg/ml of the sample were replaced with 0.1, 0.25, 0.5, 0.75, 1 mg/ml of the BHT. A lower absorbance indicates a higher radical scavenging power. DPPH scavenging activity was calculated as follows

DPPH scavenging activity (%) = $[1 – (A_t / A_o)]$ x 100

(A_t is the absorbance of the sample at 515 nm and A_o is the absorbance of the control at 515 nm).

RESULTS AND DISCUSSION

DPPH scavenging activity

DPPH radical scavenging activity of WRE was assayed to estimate its ability to react and quench DPPH radicals at selected dose levels (0.1, 0.25, 0.5, 0.75 and 1 mg/ml). There was increase in scavenging activity when the concentration of WRE increased from 0.1 to 1 mg/ml (2.72±0.007 to 20.4±0.036 %). However, the scavenging activity of WRE was significantly low at all the concentration in comparison to BHT (91.2±0.003 to 93.2±0.001 %) as depicted in Table 1.

It is well known that free radicals cause auto-oxidation of unsaturated lipids. In addition to this, antioxidants are known to interrupt the free radical chain of oxidation and to donate hydrogen from phenolic hydroxyl groups, thereby forming stable free radicals that do not initiate or propagate further oxidation of lipids. Glycowithanolides (Sitoindosides VII-X and Withaferin A), active principles of WRE are known to possess antioxidative potential (Bhattacharya *et al.*, 2000) and may account for the free radical scavenging activities (Gupta and Kar, 2002). The 2, 2'-diphenyl-1-picrylhydrazyl radical has been widely used to evaluate free radical scavenging capacity of the antioxidants. Antioxidant activity of WRE was measured by the decrease in absorbance of the DPPH radical at 515 nm. In the radical form DPPH molecule had an absorbance at 515 nm which disappeared after acceptance of an electron or hydrogen radical from an antioxidant compound to become a stable diamagnetic molecule.

In the present study, there was increase in scavenging activity with increase in the concentration of WRE. However, scavenging activity was significantly less than BHT, which was used as synthetic antioxidant. The probable reason might be that it contains less phenolic content and thus exhibited less antioxidative activity comparatively (Ashwin and Mishra, 2007).

Reducing power activity

In the present investigation, reducing power of WRE was determined in comparison to butylated hydroxytoulene (BHT) as depicted in Table 2. Reducing power (as indicated by increase in absorbance at 700nm) of *W. somnifera* was significantly less as compared to BHT (72.3 ± 0.002 to 98.8 ± 0.004) but increased with an increase in concentration of extract..

Reducing power assay determined the presence of reductants (antioxidants) in the tested samples that would reduce Fe^{3+}/ferricyanide complex to ferrous form (Fe^{2+}). Fe^{2+} can therefore be monitored by measuring the formation of Perl's Prussian blue at 700 nm. Reducing power (as indicated by increase in absorbance at 700 nm) of WRE was significantly less compared to the BHT (Table 2) but increased dose dependently with maximum activity observed at highest concentration (1 mg/ml). Phenolic compounds present in WRE may also act as reducing agents, and hydrogen donators, singhlet oxygen quenchers (Caragay, 1992; Rice –Evans, Miller, & Paganga, 1996) and electron donor (Singh *et al.,* 2006). It has been stated that phenolic groups play an important role in antioxidant activity and provide effective defense system against free radical attack (Lu *et al.,* 2001). Thus, these findings supported our investigation that WRE possess antioxidative property.

Table 1:

Radical scavenging ability of *W. somnifera* root extract (WRE) on 2, 2, diphenyl-1-picrylhydracyl radical as compared to butylated hydroxytoulene (BHT) (mean ± S.E, n = 3).

	Radical scavenging effect (%)	
Concentration (mg/ml)	WRE	BHT
0.1	2.72 ±0.007	91.2±0.003
0.25	5.60±0.045	91.8±0.008
0.5	10.2±0.028	92.3±0.001
0.75	12.5±0.032	92.7±0.00
1	20.4±0.036	93.2±0.001

Table 2:

Reducing power of *W. somnifera* root extract (WRE) as compared to butylated hydroxytoulene (BHT) (mean ± S.E, n = 3).

	Radical scavenging effect (%)	
Concentration (mg/ml)	WRE	BHT
0.1	59.4 ±0.005	72.3±0.002
0.25	63.7±0.012	81.3±0.00
0.5	67.8±0.006	87.9±0.003
0.75	72.9±0.00	94.6±0.001
1	88.3±0.009	98.8±0.004

Table 3:
Chelating effect of *W. somnifera* root extract (WRE) as compared to EDTA (0.01mM) and citric acid (0.025M) (mean ± S.E, n = 3).

Concentration (mg/ml)	Radical scavenging effect (%)		
	WRE	EDTA (0.01mM)	Citric acid (0.025)
0.1	26.5±0.007	65.2±0.003	69.2±0.006
0.25	27.5±0.013		
0.5	61.2±0.011		
0.75	40.2±0.001		
1	43.3±0.011		

Effect on the chelating activity of Fe^{+2}

Chelating activity on Fe^{+2} of WRE (0.1, 0.25, 0.5, 0.75 and 1 mg/ml) showed an increase in chelating activity on Fe^{+2} in a dose–dependent manner (Table 3). The activity was observed to be maximum at highest concentration in the present study. However, chelating activity of WRE remained less significantly for all the concentrations as compared to EDTA at 0.01mM (65.2 ± 0.003 %) and citric acid at 0.025M (69.2 ± 0.006 %).

Iron is known to be most important lipid oxidation pro-oxidant due to its high reactivity among the transition metals. The ferrous state of iron accelerates lipid oxidation by breaking down hydrogen and lipid peroxide to reactive free radicals like Fenton type reaction (Fe^{2+} + H_2O_2 '! Fe^{3+} + OH^- + OH^o). Fe^{3+} ions also produce radicals from peroxides, although the rate is tenfold less than that of Fe^{2+}. Fe^{2+} ions are the most powerful pro-oxidant among various species of metal ions (Halliwell and Gutteridge, 1984). Ferrozine, a chelating reagent, was used to indicate the presence of chelator in the reaction system. Ferrozine forms a complex with free Fe^{2+} but not with bound Fe^{2+}. In the presence of chelating agents, the complex formation of ferrous and ferrozine is disrupted, resulting in decrease in the colour of the complex. Measurement of colour reduction therefore allows estimating the metal chelating activity of the coexisting chelator (Yamaguchi *et al.*, 2000).

In this investigation, WRE showed increase in chelating effect in a dose dependent manner. Probably, WRE might have either chelated metal ions or suppressed reactivity by occupying all coordination sites of the metal ion. Therefore, WRE might have protected against oxidative damage. It is concluded from the study that the extract showed increase in DPPH scavenging property, reducing power and chelating effect on Fe^{2+} in a dose-dependent manner so i*n vitro* antioxidative activities of WRE confirmed the antioxidative property of WRE.

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Research Article

EVALUATION OF ANTIUROLITHIC ACTIVITY OF ETHANOLIC EXTRACT OF *TRIBULUS TERRESTRIS* IN WISTAR RATS

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ABSTRACT

In the present study antiurolithic activity of ethanolic extract of *Tribulus terrestris* was investigated in wistar rats. Urolithiasis was induced in animals by using ethylene glycol (0.75 % v/v) in drinking water for 28 days. The ethanolic extracts of *Tribulus terrestris* was administered @ 100, 200 and 400 mg/kg body weight orally for 28 days along with 0.75% ethylene glycol in treatment group, respectively. The mean value of urea, BUN, creatinine, uric acid, calcium and phosphorus significantly decreased in animals treated with 400 mg/kg of ethanolic extract of *Tribulus terrestris* as compared to control group. Result of the present study revealed that ethanolic extract of *Tribulus terrestris* possesses significant antiurolithic activity.

Key words: Antiurolithic activity, *tribulus terrestris,* ethylene glycol, ethanolic extract, wistar rats.

INTRODUCTION

Urolithiasis is the process of formation of stone in the urinary tract by crystal nucleation, aggregation and retention in the urinary tract. Among animals nephrolithiasis or renal stone remains a common disorder especially in the adult male bovine population resulting in lot of discomfort to the affected animals and affecting their utility. The medical management of nephrolithiasis involves cost and is also associated with side effects (Nadkarni, 1976). At presently, the treatments by surgery along with drug therapy are practiced in the management of kidney stones but have some limitations. Surgical procedures like extracorporeal shock wave lithotripsy have increased risk of stone recurrence and are also costly. Therefore, it is worthwhile to look for an alternative for the management of urolithiasis. Many medicinal plants have been employed since long back to treat urinary stones though the rationale behind their use is not well established through systematic and pharmacological studies. Recently several plants are being explored for their anti-urolithiatic properties on the basis of their usage in the traditional medicine. Therefore, search for antilithiatic drugs from natural sources has greater importance which can be used in the prophylaxis of urolithiasis.

Tribulus terrestris L. is a well-known and widely distributed species of the genus *Tribulus. T*. *terrestris* is an annual, prostrate hairy herb with many slender spreading branches with opposite, pinnately compound leaves, bright yellow flowers on slender hairy stalks. The plant is distributed throughout India in warm and hot places. It is a common weed of waste places and road sides, chiefly in hot dry or sandy localities and widely used as folk medicine in many countries for different purposes. In India, it is commonly called "Gokshur or Gokharu or puncture vine". *Tribulus terrestris* extract contains many compounds such as alkaloids, flavonoids, oil, saponins, resins and nitrates (Li *et al.*, 2002). The fruits are commonly used in traditional medicine as a diuretics (Al-Ali *et al.*, 2003). *Tribulus terestris* extracts are mainly used for kidney disorders (Gupta *et al.*, 1997). Traditionally *Gokshura* fruits are used in Ayurvedic formulations for treating urinary disorders, calculus formation, impotence and other urinary tract infection issues (Sivarajan and Balachandran,1994). Hence the present study was undertaken to investigate antiurolithic activity of ethanolic extract of *Tribulus terrestris* in ethylene glycol induced urolithiasis in rats.

MATERIALS AND METHODS

Experimental Animals

Twenty five healthy male wistar rats weighing between 150–250 g were acclimatized in the laboratory animal house of Veterinary college, AAU, Anand for 5 days before experiment commenced. They were kept at 22 ± 2°C with a controlled photo period of 12 hours light and 12 hours darkness. Every individual animal was provided conventional laboratory pellet diet with *ad libitum* drinking water. Animal housing, environmental conditions, grouping of animals, animal numbering and identification of each animal were as per standard procedure. The experiment was designed and conducted in accordance with the guidelines of Institutional Animal Ethical Committee (No. IAEC/2010/VPT/79).

Experimental design

All rats were randomly divided into five groups having five animals in each. Rats of vehicle control group

received orally 0.5% sodium bicarbonate in Milli-Q water. Lithiatic control rats received 0.75% v/v ethylene glycol in drinking water for 28 days in drinking water. Rats of treatment group received ethanolic extracts of *Tribulus terrestris* @ 100, 200 and 400 mg/kg body weight daily once orally for 28 days along with 0.75% ethylene glycol in drinking water, respectively.

Preparation of ethanolic extract of *Tribulus terrestris* & administration

Tribulus terrestris plants were procured from their natural habitat and were identified and authenticated by Botanist, Department of Genetics and Plant Breeding, B.A. College of Agriculture, AAU, Anand. The fruits of the plant were dried in shade. The dried fruits were pulverized in an electric grinder to get a coarse powder. The dried plant material was subjected to repeated extraction in Soxhlet apparatus using ethanol. The extract was then concentrated (dark greenish brown, semi-solid, peculiar odor) using rotary evaporator under reduced pressure. The ethanolic extract was stored in a labeled sterile screw capped glass bottle at -60°C until experimental uses. Ethanolic extract of *Tribulus terrestris* was dissolved in 0.5% sodium bicarbonate solution for oral administration. Dose were calculated according to body weight of each animal and administrated as per concentration strength of formulation.

Acute oral toxicity testing

The acute oral toxicity study was carried out as per the guideline No. 423 set by Organization for Economic Cooperation and Development (OECD). Wistar rats were taken for the study and dosed once with 2000 mg/kg, orally. The treated animals were monitored for 24 hours and then up to 14 days for general clinical signs and symptoms like salivation, lacrimation and urination as well as mortality. It was observed that the test extract did not produce mortality in rats at 2000 mg/kg dose. Hence, 100, 200 and 400 mg/kg of this Plant extract dose were selected for further study.

Ethylene glycol induced urolithiasis model

For induction of urolithiasis, 0.75% v/v ethylene glycol (Merck Limited, Mumbai) in drinking water for 28 days was given *ad-libitum* in drinking water.

Assesment of anti-urolithiatic activity

Anti-urolithiatic activity of repeated oral administration of ethanolic extracts of *Tribulus terrestris* were assessed by studying haematological parameters, serum biochemical parameters and histopathological examination of tissues. Body weights and feed consumption were also recorded at weekly interval till day 28 of the experiment period. A careful physical and behavioral examination of all rats was carried out during experimental period.

Collection of blood samples

On 28th day of experiment, blood samples were collected from the retro-orbital plexus under light surgical anesthesia into clean sterilized plain and K_3EDTA added micro-centrifuge tube for serum biochemical and hematological analysis, respectively.

Hematological estimation were carried out for the assessment of parameters like RBCs, haemoglobin, packed cell volume, MCV, MCH, MCHC, WBCs, lymphocyte %, neutrophil % and platelets counts using automated haematology analyzer (Abacus Junior Vet 5, Austria). Collected blood samples for serum biochemical analysis were allowed to clot and then centrifuged at 3000 rpm for 10 minutes to seperate serum. Serum biochemical parameters were analyzed using automatic serum biochemical analyzer (Junior Selectra Vital Scientific NV, Netherlands). Serum biochemical parameters like alanine amino transferase (ALT), aspartate amino transferase (AST), alkaline phosphatase (ALP), urea, blood urea nitrogen (BUN), creatinine, uric acid, calcium, phosphorus and magnesium were quantified by using standard assay kits (Merck Specialities Pvt. Ltd., Goa, India).

Statistical analysis

The data obtained for body weight, feed intake, hematological parameters and serum biochemical parameters were subjected to statistical analysis (unpaired two tail 't' test). Where p d" 0.05 was considered as statistically "significant" and p d" 0.01 was considered as statistically "highly significant".

RESULTS AND DISCUSSION

All rats were observed daily throughout the period of study. All the rats were found active during experimental period and did not reveal any abnormal symptoms attributable to the 28 days oral administration of ethanolic extracts of *Tribulus terrestris*. There was no mortality during study period.

In the present study, daily oral administration of 0.75% v/v ethylene glycol in drinking water for 28 days was given to establish urolithiasis in wistar rats based on serum biochemical and histopathological evaluation. Induction of urolithiasis was manifested by increased in stone forming constituents like urea, BUN, creatinine, uric acid, calcium and phosphorus as compared to vehicle control rats. The histopathological studies also confirmed the induction of urolithiasis. Several studies have been reported on induction of urolithiasis by ethylene glycol alone or in combination with other crystal-inducing drugs such as ammonium chloride or Vitamin D3 (Halabea *et al.*, 2003), gentamicin or a magnesium deficient diet (Jie *et al.*, 1999), gentamicin sulphate @ 40 mg per kg body weight intraperitonial daily for 14 days (Sejal *et al.*, 2015). In the present study, ethylene glycol was used for producing urolithiasis in male Wistar

Table 1:
Effect of *Tribulus terrestris* ethanolic extract on body weight of Wistar Rats (Mean ± SE, n=5)

Group	Day 1	Day 7	Day 14	Day 21	Day 28
Vehicle Control	235.00±2.89	238.33±1.67	236.67±3.33	238.33±6.01	243.33±3.33
Lithiatic Control	226.67±12.02	226.67±16.67	243.33±8.82	241.67±13.02	238.33±8.33
TT Ethanolic extract100mg/kg	223.33±12.02	233.33±12.02	230.00±15.28	228.33±9.28	231.67±15.90
TT Ethanolic extract200mg/kg	243.33±8.82	246.67±6.67	246.67±10.14	243.33±8.82	253.33±8.82
TT Ethanolic extract400mg/kg	250.00±11.55	250.00±11.55	243.33±17.64	253.33±13.02	256.67±14.53

Table 2:
Mean feed consumption/ day/ rat in *Tribulus terrestris* ethanolic extract treated groups (n=5)

Group	Week 1	Week 2	Week 3	Week 4
Vehicle Control	12.50	13.07	11.43	13.86
Lithiatic Control	11.90	13.10	11.86	11.95
TT Ethanolic extract100mg/kg	10.36	11.07	12.50	15.36
TT Ethanolic extract200mg/kg	11.43	13.21	11.43	16.43
TT Ethanolic extract400mg/kg	10.71	11.79	11.79	16.07

Table 3:
Effect of *Tribulus terrestris* ethanolic extract on hematological parameters in wistar rats (Mean ± SE, n=5)

Group	RBC	Hb	PCV	MCV	MCH	MCHC	WBC	Lymphocytes	Neutrophils	Platelets
	10^6/µl	g/dl	%	Fl	Pg	g/dl	10^3/µl	%	%	10^5/µl
Vehicle Control	7.71±0.02	14.60±0.17	41.88±0.33	54.00±0.58	18.95±0.26	34.80±0.12	7.58±.27	79.40±1.04	14.05±0.55	4.03±0.18
Lithiatic Control	6.71±0.75	12.47±0.63*	36.14±3.63	57.33±3.93	17.80±0.53	32.97±044*	9.13±1.17	79.10±3.76	16.30±3.25	5.34±031*
TT Ethanolic extract 100mg/kg	7.46±0.08	13.60±0.06	40.24±0.44	54.00±1.15	18.20±0.12	27.63±3.44	6.73±0.88	77.47±2.03	17.53±0.37	4.29±0.70
TT Ethanolic extract 200mg/kg	5.42±0.84	13.33±2.60	31.30±4.38	55.33±0.33	17.80±0.76	32.10±1.15	7.57±1.23	81.00±4.52	12.40±0.95	4.88±0.59
TT Ethanolic extract 400mg/kg	6.23±0.49	12.50±0.40	35.07±2.88	56.50±0.29	20.35±0.95	34.15±1.00	5.94±0.54	72.88±1.74	19.70±5.08	4.47±0.53

* Significant difference ($p< 0.05$), RBC: Red blood cells; Hb: Haemoglobin; PCV: Packed cell volume; MCV: mean corpuscular volume; MCH: mean corpuscular hemoglobin; WBC: White blood cells.

Table 4:
Effect of *Tribulus terrestris* ethanolic extract on serum biochemical parameters in wistar rats (Mean ± SE, n=5)

Group	ALT	AST	AKP	TP	UREA	BUN	Creatinine	Uric acid	Ca	P	Mg
	IU/L	IU/L	IU/L	g/dl	mg/dl	mg/dl	mg/dl	mg/dl	mg/dl	mg/dl	mg/dl
Vehicle Control	43.78±1.98	84.68±3.29	168.35±15.33	6.68±0.44	51.78±2.08	24.18±0.97	0.53±0.07	1.82±0.28	9.52±0.04	5.06±0.07	3.11±0.01
Lithiatic Control	71.33±5.55*	84.10±7.00	170.27±31.51	6.92±0.29	97.60±1**	45.58±0.47**	0.97±0.12*	3.63±0.08**	13.01±0.41**	8.56±0.40	3.71±0.65
TT Ethanolic extract 100mg/kg	73.17±2.80	79.00±2.89	210.43±4.23	6.93±0.29	89.13±3.35	42.63±1.56	0.68±0.12	2.68±0.89	10.61±0.97	8.30±0.06	2.35±0.33
TT Ethanolic extract 200mg/kg	53.15±6.38	84.57±1.75	195.80±16.34	6.94±0.16	89.37±2.87	42.67±1.34	0.65±0.14	2.38±0.44	10.20±1.01	7.93±0.18	3.00±0.01
TT Ethanolic extract 400mg/kg	50.80±2.77	57.25±0.38*	138.60±1.79	6.84±0.55	61.55±9.68*	28.74±4.52*	0.55±0.09*	2.27±0.23**	10.10±0.07*	6.75±0.76*	3.03±0.01

* Significant difference ($p< 0.05$), ** highly significant difference ($p< 0.01$), ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; AKP: Alkaline phosphatase; TP: Total protein; BUN: Blood urea nitrogen; Ca: Calcium, P: Phosphorus; Mg: Magnasium

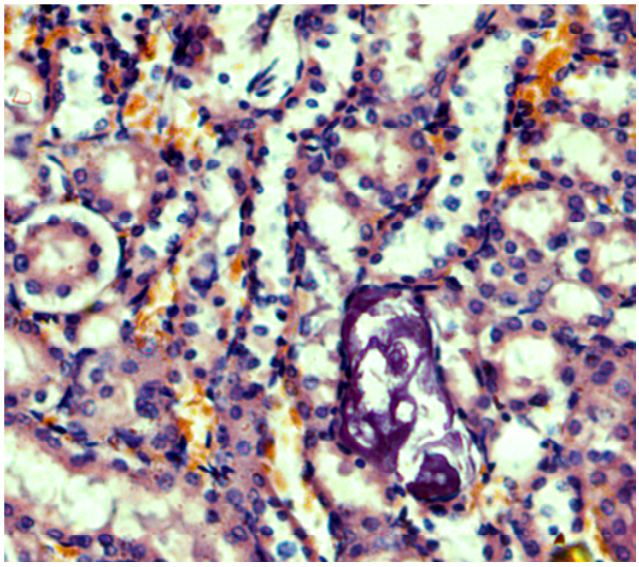

Fig.1:
Lithiatic control group showing crystals deposition in renal tubules along with cast, severe degeneration and necrosis of renal tubular epithelium

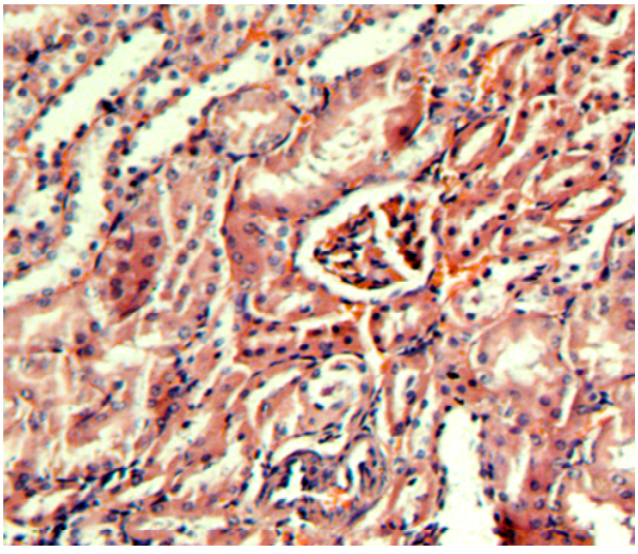

Fig.2:
Tribulus terrestris ethanolic extract treated (400mg/kg) rats showing mild degeneration and necrosis of renal tubular epithelium

rats. The principal target organ following oral exposure to ethylene glycol is the kidney; moreover, evidences from previous studies indicated that administration of ethylene glycol caused renal stone formation by increasing hyperoxaluria (Green *et al.*, 2005). In the present study, male rats were selected to induce urolithiasis because the urinary system of male rats resembles that of humans and few animals, and previous studies have shown that the amount of stone deposition in female rats was significantly less as compared to male. Previous studies have reported that young male albino rats form renal calculi composed mainly of calcium oxalate in response to 14 day period of ethylene glycol (0.75%, v/v) administration (Selvam *et al.*, 2001).

Body weights and feed intake were recorded at weekly interval till day 28 of the experiment period. There was non-significant change in body weight as well as feed consumption of rats of treatment groups as compared to lithiatic control group and are presented in Table 1 and 2. Similarly, Sadariya *et al.* (2014) also reported that following repeated oral administration of aqueous extract of *Tribulus terrestris* at dose of 100, 200 and 400 mg/kg body weight did not showed any significant alteration in body weight and feed consumption as compared to control rats.

Values of hematological parameters of treatment and control rats are presented in Table 3. The mean values of Hb and MCHC were significantly decreased and platelet count was significantly increased in lithiatic control group as compared to corresponding vehicle control group. The hematological parameters of rats treated with 100, 200 and 400 mg/kg of ethanolic extracts for 28 days did not differ significantly ($P < 0.05$) from the corresponding values observed in lithiatic control rats. Likewise, Sadariya *et al.* (2014) also reported that following repeated oral administration of aqueous extract of *Tribulus terrestris* at dose of 100, 200 and 400 mg/kg body weight did not showed any significant alteration in hematological parameters as compared to control rats.

Mean values of serum biochemical parameters of treatment and control groups were presented in Table 4. In the results of present study, mean values of ALT, urea, BUN, creatinine, uric acid, calcium and phosphorus were significantly increased in lithiatic control rats as compared to vehicle control rats. This elevated serum levels are indicative of the marked renal damage in urolithiasis induced rats. Whereas, mean values of AST, urea, BUN, creatinine, uric acid, calcium and phosphorus were significantly decreased in 400 mg/kg of ethanolic extract of *Tribulus terrestris* treated rats as compared to lithiatic control rats. Similarly, Saxena and Argal (2015) reported antiurolithiatic activity of a herbal suspension containing alcoholic extracts of fruit of *Tribulus terrestris*, root of *Boerhavia diffusa* and leaves of *Azadirachta indica* in ethylene glycol-induced urolithiasis model in wistar rats. The herbal suspension decreased the level of calcium, oxalate and phosphate significantly at doses of 200 and 300 mg/kg as compared to the control group. Creatine, uric acid and urea were also decreased significantly at 100, 200 and 300 mg/kg dose levels in rats. Increased urinary calcium is a factor favoring the nucleation and precipitation of calcium oxalate or calcium phosphate from urine and subsequent crystal growth results in stone formation (Robertson and Peacock, 1980). Increased urinary phosphate excretion along with oxalate stress seems to provide an environment appropriate for stone formation by forming calcium phosphate crystals, which induces calcium oxalate deposition (Gitelman, 1967). But treatment with ethanolic extract of *T. terrestris* restored the phosphate level, thus reducing the risk of stone formation. Urolithiasis also decreases the glomerular filtration rate due to obstruction in outflow of urine by stones in the urinary system. Due to this, waste products, particularly nitrogenous substances such as urea, creatinine, and uric acid are accumulated in the blood (Ghodkar, 1994). Uric acid is also known to promote calcium oxalate crystal growth because of predominance of uric acid crystals in calcium oxalate stones (Roger *et al*, 1997). The curative and prophylactic treatment with *Tribulus terrestris* ethanolic extracts causes diuresis which hastens the process of dissolving the preformed stones, increased excretion of urea, creatinine and uric acid as well as normalized the kidney functions and prevents the formation of new stone in the urinary system (Singh *et al.*, 1991).

All the rats were subjected to postmortem examination on day 29 in the confined disinfected laboratory to determine the presence or absence of gross and histopathological lesions. For gross lesions liver, kidney, heart and spleen were collected and examined after opening the body of sacrificed experimental rats. Gross pathological examination did not revealed any abnormal lesions attributable to the 28 days oral administration of ethanolic extracts of *Tribulus terrestris* in all three treatment groups.

For histopathological examinations, tissues from liver, kidney, heart and spleen were collected and processed as per standard methods and sections were stained with haematoxylin and eosin (H & E) and examined for microscopic lesions. Ethylene glycol induced urolithiasis group revealed presence of polymorphic irregular crystals in renal tubules accompanied by cast formation which causes dilation of proximal tubules. Severe degeneration and necrosis of renal tubular epithelium were also observed (Figure 1). These histological observations support the presence and growth of renal calculi in kidney as observed in

urolithiasis. Ethanolic extracts (200 and 400 mg/kg) of *Tribulus terrestris* treated group revealed moderate to few crystals along with mild degeneration and necrosis of renal tubules. Presence of few crystals in the renal tubules indicates the ability of ethanolic extract (400 mg/kg) of *Tribulus terrestris* to dissolve the pre-formed stones (Figure 2). There were no changes in cellular structures and no other abnormal microscopic lesions in liver, heart and spleen of all treated and control rats. Histopathological examination is also supported by the biochemical parameters observed from higher dose of ethanolic extract of *Tribulus terrestris* (400 mg/kg) decrease the development of nephrotoxicity. Overall, the data presented in this research paper indicates that repeated oral administration of ethanolic extracts of *Tribulus terrestris* for 28 days to experimentally ethylene glycol induced urolithiasis rats reduced the deposition of crystals in kidneys, confirming its antilithiatic effect.

Our study was well supported by the report of the anticalcifying proper-ties of *Tribulus terrestris* on calcium oxalate crystal nucleation and growth *in vitro* as well as fur-ther examining the potency of *Tribulus terrestris* on oxalate induced injury in NRK 52E (rat renal tubular epithelial) cells (Aggarwal *et al.*, 2010). Kavitha and Jagadeesan (2006) also reported that the oral administration of methanolic fraction of *Tribulus terrestris* fruit extract at dose 6 mg/kg body weight provided protection against the mercuric chloride induced toxicity in the mice. Similarly, the ethanol extract of *T. terrestris* (fruit) administered at daily oral doses of 25, 50 and 100 mg/kg for 4 months exhibited dose-dependent antiurolithiatic activity and almost completely inhibited stone formation in rats. Other biochemical parameters in urine and serum and the histopathology of urinary bladder, which were altered during the process of stone formation, were also normalized by the plant extract in a dose-dependent manner (Anand *et al.*, 1994).

Repeated oral administrations of ethanolic extracts of *Tribulus terrestris* @ 400 mg/kg daily once for 28 days were effective against ethylene-glycol induced urolithiasis in rats. Our study suggests the possibility of using *Tribulus terrestris* ethanolic extract as a prophylactic alternative to prevent urolithiasis.

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Research Article

PHARMACOKINETICS OF SULFAQUINOXALINE FOLLOWING SINGLE DOSE ORAL ADMINISTRATION IN POULTRY

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ABSTRACT

The present study was undertaken to investigate the pharmacokinetics and tissue residue study of sulfaquinoxaline in poultry following single dose (200 and 50mg.kg^{-1}) oral administration. The concentration of sulfaquinoxaline in plasma and tissues of poultry birds was analyzed by HPLC. The initial peak plasma concentration of 43.845 and 41.369 µg.ml^{-1}was detected in poultry following single dose 200mg.kg^{-1} and 50mg.kg^{-1} oral administration, respectively. The volume of distribution, clearance, area under curve (AUC), elimination half- life, T_{max} and C_{max} calculated were 1869.75 ml.kg^{-1}, 55.488 ml.h^{-1}.kg^{-1}, 3808.429 µg.ml^{-1}.h, 34.456 h, 19.87 h and 50.813 µg.ml^{-1}, respectively, following single dose (200mg.kg^{-1})and 632.017 ml.kg^{-1}, 29.881 ml.h^{-1}.kg^{-1}, 1692.282 µg.ml^{-1}.h, 20.339 h, 8.081 h and 43.473µg.ml^{-1}, respectively, following single dose (50mg.kg^{-1}) oral administration of sulfaquinoxaline. Tissue residue studies revealed that after single dose (200 and 50mg.kg^{-1}) oral administration, sulfaquinoxaline, residues were detected in liver, kidney, muscle, intestine, fat and skin upto 120 h and 96 h, respectively with the highest concentration detected in muscle. Therefore, based upon the present study, a priming dose of 40mg.kg^{-1}followed by a maintenance dose of 14 mg.kg^{-1} is recommended. Withdrawal periods of 6 and 7 days, respectively are recommended after single dose (200mg.kg^{-1}) and single dose (50mg.kg^{-1}) oral administration of sulfaquinoxaline.

INTRODUCTION

Sulfonamides were the first drugs with a selective effect on bacteria, and which could be used systemically against bacterial infections (Domagk, 1935). More than 5000 derivatives of sulfanilamide were synthesized in the subsequent years but approximately 20 derivative forms are generally available. The spectrum of activity for the sulfonamides is broad, affecting gram-positive, gram-negative and many protozoan organisms and are bacteriostatic rather than bactericidal. They are used to treat infections of CNS, respiratory tract, gastrointestinal tract (among a variety of other soft tissues), and, in particular, the urinary tract. In the veterinary field, sulfonamides were proposed for treatment and, in the past, as growth promoters for some decades (Plumb, 2005). They are reportedly effective against a wide variety of infectious diseases and commonly therapeutic especially for cattle and swine. In poultry, sulfonamides have been used for treatment of coccidiosis, fowl cholera, infectious coryza, pullorum disease and fowl typhoid (Giguère *et al.*, 2006). The drug is still being used commonly in poultry sector thus, making it essential to conduct pharmacokinetic and tissue residues study.

MATERIAL AND METHODS

Experimental animals

The study was conducted in Rhode Island Red poultry birds aged 3-4 months and weighing 1.5±0.5 kg. Birds were procured from Instructional poultry farm (IPF) of the University and kept in cage system in the animal house of the College throughout the study period. They were kept for acclimatization for a period of one month prior to initiation of the experiment and were dewormed with albendazole (10% albendazole oral suspension) during this period. The birds were reared under uniform management and husbandry conditions, maintained on standard ration free of antibiotic and provided water *ad libitum*. All the birds were reared as per the ethical guidelines of CPCSEA with permission from IAEC.

Sulfaquinoxaline (Supercox™) was administered at single dose (200 and 50mg.kg-1) p.o. to two separate groups of poultry birds having six birds in each group.

Blood samples following single dose (50 mg.kg^{-1}) were collected from six birds in heparinized tubes through an i.v. cannula placed in either contralateral jugular veins or wing vein at 0, 5, 15, 30 min. & 1, 2, 4, 8, 12, 24, 48, 72 and 96 h after drug administration Blood samples were collected from six poultry birds at 0, 5, 15, 30 min. & 1, 2, 4, 8, 12, 24, 48, 72, 96, 120, 144, 168 and 192 h following single dose (200 mg.kg^{-1}) administration.

Tissue residue study was conducted in five groups of birds, each comprising three birds. Groups were administered sulfaquinoxaline as single oral dose @ 50mg.kg^{-1} and tissues viz., liver, kidney, muscle, intestine, fat and skin were collected from the birds. Birds were sacrificed humanely at 24 h (Group I), 48 h (Group II), 72 h (Group III), 96 h (Group IV) and 120 h (Group V), respectively, post sulfaquinoxaline administration.

Tissue residue study was conducted in seven

groups of birds, each comprising three birds. A single oral dose (200mg.kg^{-1}) of sulfaquinoxaline was administered to birds different tissues viz., liver, kidney, muscle, intestine, fat and skin were collected. Tissues were collected from Group I (control) birds for calculation of recovery percentage of sulfaquinoxaline. Birds were sacrificed at 24 h (Group II), 48 h (Group III), 72 h (Group IV), 96 h (Group V), 120 h (Group VI) and 144 h (Group VII), respectively, post sulfaquinoxaline administration.

Drug extraction from plasma samples was carried out as per the method of Suber RL and Edds GT (1980) with slight modifications. Drug extraction of sulfaquinoxaline from tissues was carried out by the method as described by Ioerger and Smith (1993) with modifications. 4 g of frozen tissue was thawed and triturated in a mortar. Double amounts of methanol was added to the triturated tissue and subjected to homogenization to make a paste. The paste was subjected to sonication at 10 amplitude microns for 30 sec, with a pause of 5 seconds (a total of 15 cycles) by using ultrasonic tissue disintegrator. The sonicated tissue was centrifuged at 9000 rpm for 15 min. The supernatant was collected in petridish and dried at 37°C. Clean up was done by the method of Telling and Sissons (1977) with slight modifications.

The estimation of Sulfaquinoxaline in plasma and tissue was done by high performance liquid chromatography as per the method of Suber RL and Edds GT (1980) with modifications. HPLC conditions were kept similar for plasma and tissue samples. An isocratic mobile phase consisted of 50% of water and 50% methanol HPLC grade, pH adjusted to 4 using glacial acetic acid . The flow rate was kept at 0.5 mL.min^{-1}. Chromatography was performed at 25^0 C with UV detection at 254 nm. The chromatogram was analyzed by a pharmacokinetic software. The limit of quantification (LOQ) of sulfaquinoxaline was 0.025 mg.ml^{-1} and the limit of detection (LOD) was 0.05 mg.ml^{-1}.

Recovery of sulfaquinoxaline residues was calculated by employing the following formula:

$$\text{Recovery \%} = \frac{N(\sum xy)-(\sum x)(\sum y)}{N(\sum x^2)-(\sum x)^2} \times 100$$

Where, x = amount of standard drug added, y = amount of drug found by proposed method, N = number of observations.

Table 1:
Comparative pharmacokinetic parameters of sulfaquinoxaline in plasma following single dose (50mg.kg^{-1} and 200mg.kg^{-1}) oral administration in poultry (n=6).

Parameters	Units	Mean±S.E.	
		50mg.kg^{-1}	200mg.kg^{-1}
K01	1/h	0.255±0.054*	0.083±0.009
K10	1/h	0.055±0.005	0.032±0.001
K01_HL	h	2.454±0.431	7.138±0.955**
K10_HL	h	20.339±0.998	34.456±2.852**
AUC	µg.ml-1.h	1692.282±71.835	3808.429±310.978**
V_F	ml.kg-1	632.017±55.020	1869.750±242.345**
CL_F	ml.h-1.kg-1	29.881±1.134	55.488±5.220**
Tmax	h	8.081±0.880	19.870±1.812**
Cmax	µg.ml-1	43.473±1.424	50.813±1.768**

Values bearing superscript (*) differ significantly (p<0.05)
Values bearing superscript (**) differ significantly (p<0.01)
K01:First order absorption rate constant in one compartment model, K10:First order elimination rate constant in one compartment model, K01_HL: Absorption half life in one compartment model K10_HL: Elimination half life in one compartment AUC: Total area under the time concentration curve, C_{max}: Peak plasma concentration, T_{max}: Maximum time required to attain peak plasma concentration; Cl_F: Total body clearance, V_F :Volume of distribution.

RESULTS AND DISCUSSION

A one-compartment model (r = 0.987) adequately described plasma concentration time profile of sulfaquinoxaline in poultry following single dose (50 mg.kg^{-1} and 200 mg.kg^{-1}) oral administration. The plasma concentration-time profile following single dose (50mg.kg^{-1}) oral administration of sulfaquinoxaline in poultry is depicted in Figure 1. The mean peak plasma concentration of 41.369±0.824 mg.ml^{-1} was attained at 8 h post administration and the minimum detectable level was estimated to be 0.610±0.113 mg.ml^{-1} at 96 h. Thereafter, concentration of sulfaquinoxaline was undetectable in plasma.

In the present study, the first order absorption rate constant (K_{01}) was 0.255 h^{-1} which is lower as compared to 0.64 h^{-1} reported in laying hens after oral dosing of 100mg.kg^{-1} sulfadimidine (Tansakul *et al.*, 2008). Absorption half-life (K_{01}_HL) in the present study was estimated to be 2.454 h. This value can be correlated with the value of 1.2 h reported in laying hen following oral administration (100mg.kg^{-1}) of sulfadimidine (Tansakul *et al.*, 2008).

The first order elimination rate constant (K_{10}) in the present study was 0.055 h^{-1} with an elimination half-life (K_{10}_HL) of 20.339 h. However, El-Sayed *et al.* (1995) have reported an elimination half-life of 12.6 h in broilers following i.v. administration (100mg.kg^{-1}) of sulfaquinoxaline while Jain and Punia, 2001 reported 6.1 h in poultry following oral administration of (100mg.kg^{-1}) sulphadimidine.

The AUC value of sulfaquinoxaline in poultry was calculated to be 1692.282 µg.ml^{-1}.h in the present study which is slightly higher as compared to 1379 µg.ml^{-1}.h as reported by Tansakul *et al.* (2008) in laying hens following oral administration (100mg.kg^{-1}) of sulfadimidine, respectively. Volume of distribution in the present study was estimated to be 632.017 ml.kg^{-1}.El-Sayed *et al.* (1995) have reported volume of distribution at steady state (Vdss) of 440 ml.kg^{-1} following i.v. (100mg.kg^{-1}) administration of sulfaquinoxaline in broilers. Clearance was measured to be 29.881 ml. h^{-1}.kg^{-1} which is lower as compared to the value (82.47

Table 2:
Residual concentration (µg.g^{-1}) of sulfaquinoxaline in various tissues following single dose (50 mg.kg^{-1} and 200mg.kg^{-1}) oral administration in poultry at different time intervals.

Tissues	Mean ± S.E. (n=3)						
	Doses	24h	48h	72h	96h	120h	144 h
Liver	50	1.570±0.427	0.602±0.062	0.817±0.035	0.181±0.007	ND	-
	200	4.100±0.399	3.110±0.185	2.043±0.068	0.774±0.240	0.277±0.005	ND
Kidney	50	4.063±0.625	4.141±0.079	1.914±0.274	ND	ND	-
	200	6.065±0.202	4.138±0.215	1.905±0.261	1.396±0.143	0.461±0.138	ND
Muscle	50	8.576±0.437	4.816±0.368	2.840±0.253	ND	ND	-
	200	13.425±0.204	9.116±0.170	7.978±0.288	5.015±0.737	1.894±0.300	0.440±0.041
Intestine	50	1.162±0.134	0.691±0.066	0.338±0.045	ND	ND	-
	200	4.241±0.158	2.925±0.108	1.752±0.237	0.303±0.040	ND	ND
Fat	50	2.907±0.296	2.748±0.235	0.676±0.056	ND	ND	-
	200	4.061±0.195	2.160±0.014	1.132±0.186	0.345±0.102	0.047±0.000	ND
Skin	50	4.855±0.557	3.211±0.276	0.523±0.040	0.305±0.101	ND	-
	200	5.110±0.273	2.875±0.299	1.459±0.170	0.803±0.279	0.137±0.039	ND

Study not conducted, ND: Not Detectable

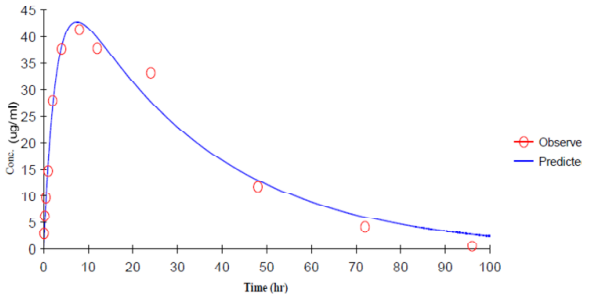

Fig. 1:
Plasma concentration – time plot of observed (mean) *vs.* predicted profile of sulfaquinoxaline following single dose (50 mg.kg^{-1}) oral administration in poultry (n=6).

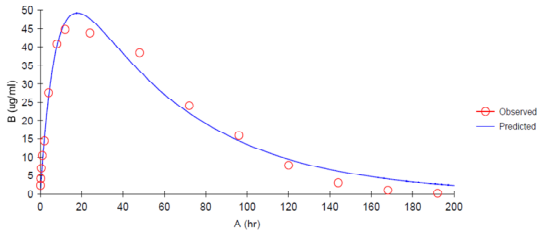

Fig. 2:
Plasma concentration – time plot of observed (mean) *vs.* predicted profile of sulfaquinoxaline following single dose (200mg.kg^{-1}) oral administration in poultry (n=6).

ml.h^{-1}.kg^{-1}) reported by Tansakul *et al.* (2008) in hens following single dose (100mg.kg^{-1}) oral administration of sulfadimidine and much higher than the value 16.68 ml.h^{-1}.kg^{-1} reported by El-Sayed *et al.* (1995) following i.v. (100mg.kg^{-1}) administration of sulfaquinoxaline to broilers.

Time required to reach the maximum concentration in plasma (T_{max}) was estimated to be 8.088 h in the present study which is higher than 5.56 h reported in broilers following oral (100 mg.kg^{-1}) administration of sulfaquinoxaline (El-Sayed *et al.*, 1995). Maximum mean plasma concentration (C_{max}) was 43.473 µg.ml^{-1} which is lower as compared to 107.8 µg.ml^{-1}, reported in broilers following oral (100mg.kg^{-1}) administration of sulfaquinoxaline (El-Sayed *et al.*, 1995), 83 µg.ml^{-1} after oral (100mg.kg^{-1}) dosing of sulfadimidine (Jain and Punia, 2001), 132.2 µg.ml^{-1},76.5 µg.ml^{-1}, 25.32 µg.ml^{-1} in laying hens following i.v. (100mg.kg^{-1}), oral (100mg.kg^{-1}) and oral (30mg.kg^{-1}) administration of sulfadimidine, respectively (Tansakul *et.al.*, 2008).

Following single dose 200 mg.kg^{-1}, the mean sulfaquinoxaline concentration at 0.08 h post administration was 2.403 mg.ml^{-1} which increased gradually to the peak plasma level of 44.792 mg.ml^{-1} at 12 h and then decreased slowly to a minimum of 0.275 mg.ml^{-1} at 192 h. In the present study, the absorption rate constant (K_{01}) was 0.083 h^{-1} which is quite similar to the corresponding value of 0.076 h^{-1} observed by Kietzmann (1980) in 5 wk. old broiler fowls after oral administration of 150mg.kg^{-1} of sulfathiazole. Absorption half-life (K_{01}_HL) was calculated to be 7.138 h which could be compared with the corresponding value of 5.12 h following administration of 100mg.kg^{-1} of sulfadimidine in broiler fowl (Kietzmann, 1980). The first order elimination rate constant (K_{10}) was 0.032 h^{-1} and the elimination half- life (K_{10}_HL) was estimated to be 34.456 h. However, Li *et al.* (1995) have reported an elimination half-life of 11.71 h in broilers following oral dosing of 200mg.kg^{-1} sulfaquinoxaline.

The AUC value (3808.429 µg.ml^{-1}.h) of sulfaquinoxaline in poultry in the present study was higher than the value (2108 µg.ml^{-1}.h) reported by Li *et al.* (1995) in broilers following oral dosing (200mg.kg-1) of sulfaquinoxaline. The apparent volume of distribution (*Vd*) indicates the apparent space (ml/kg) in the body available to contain the drug. Thus, *Vd* is the apparent distribution which relates the plasma concentration of a drug to the total amount of drug in the body at any time

after pseudo distribution equilibrium has been attained. Volume of distribution (V_F) in the present study was 1869.750 ml.kg^{-1}.

Plasma clearance is a better index of efficiency of drug elimination than half-life as it gives the clearance of drug from blood per unit of time. In the present study, clearance was 55.488 ml.h^{-1}.kg^{-1}. Time required to reach maximum plasma concentration (T_{max}) was 19.870 h which is higher than the value (3.75 h) reported by (Li *et al.*, 1995) following oral dose (200mg.kg^{-1}) administration of sulfaquinoxaline in broilers. The values of time to reach maximum plasma drug level of sulfathiazole (1-2 h) and sulfadimidine (4-8 h) reported by Kietzmann (1980) in broilers are also lower than the value calculated in the present study. The maximum mean plasma concentration (C_{max}) of 50.813 µg.ml^{-1} was achieved after single dose (200mg.kg^{-1}) oral administration of sulfaquinoxaline in poultry. This can be well correlated with the value reported in case of sulfathiazole (46.37 µg.ml^{-1}) by Kietzmann (1980).

A comparison of plasma concentrations of sulfaquinoxaline at different time intervals following single dose (200 and 50 mg.kg^{-1}) oral administration in poultry birds revealed that the plasma concentration at 24, 48, 72 and 96 h was significantly (P<0.01) higher at the dose rate of 200mg.kg^{-1}as compared to 50mg.kg^{-1} (Figure 1 & 2).

Comparative analysis of pharmacokinetic parameters at two doses (200 and 50 mg.kg^{-1}) revealed that the absorption rate constant was significantly (P<0.01) higher in birds administered sulfaquinoxaline @ 50mg.kg^{-1} as compared to 200mg.kg^{-1}. However, kinetic parameters viz. (K_{01}_HL), (K_{10}_HL), AUC, V_F, Cl_F, T_{max} and C_{max} were significantly (P<0.01) higher in birds administered 200mg.kg^{-1} of sulfaquinoxaline as compared to 50mg.kg^{-1} (Table 1).

Higher plasma concentrations at 50mg.kg^{-1} as compared to 200mg.kg^{-1} till 8 h, post administration of drug can be attributed to a higher absorption rate constant and lower absorption half-life at 50mg.kg^{-1}.

Based on the pharmacokinetic data obtained following single dose (50mg.kg^{-1}) oral administration in poultry, dosage regimen was calculated with the therapeutic concentration of 40 µg.ml^{-1} (Jain, S K. and Punia, J. S., 2001) at dosing intervals of 8, 12 and 24 hrs. A priming dose of 39.25, 48.91and 94.63 mg.kg^{-1} with maintenance dose of 13.97, 23,63 and 69.43 mg.kg^{-1}, respectively was calculated for the selected dosing intervals.(Cssmin = 39.94, 23.61, 8.045: Cssmax = 62.02, 45.68, 30.02, respectively).

Tissue residue study

The mean residual concentrations are depicted in Table 2. Sulfaquinoxaline accumulated in liver, muscle, kidney, fat and skin with highest accumulation in muscle followed by kidney. On the basis of tissue residue concentration of sulfaquinoxaline, a withdrawal period of 6 days is recommended following single dose administration @ 50mg.kg^{-1} and 7 days both for single dose administration @ 200mg.kg^{-1}.

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Research Article

EFFECT OF *WITHANIA SOMNIFERA* ROOT EXTRACT AGAINST MONOCROTOPHOS -INDUCED ALTERED RESPONSIVENESS OF CHICKEN DUODENUM TO ACETYLCHOLINE

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ABSTRACT

The contractile responses of isolated duodenal segments from chickens treated with monocrotophos (MCP) alone and in combination with *Withania somnifera* root extract (WRE) were studied using 4 channel polygraph (Biopac, USA). The study consisted of 5 groups with 12 birds in each group. Group I served as control. Monocrotophos (2 ppm in feed) was fed to chickens of group II to V and WRE (@ 100, 300 and 500mg/kg b. wt/day) was given in drinking water to birds of group III to V, respectively for 7 weeks. The cumulative doses of acetylcholine (0.01-300 μM) caused dose-dependent contractions with significant increase in E_{max} and decrease in EC_{50} values in MCP-exposed group II birds. Dose-response curve of ACh in group II birds was shifted to left. WRE significantly decreased responses of ACh in duodenum of chickens of group III to V. These findings demonstrated that MCP increased the responsiveness of muscarinic receptors in chicken duodenum and WRE had protective efficacy against MCP-induced alterations in responsiveness of cholinergic receptors to ACh in the tissue preparations.

Key words: Monocrotophos, acetylcholine, intestinal tissues, *Withania somnifera*

INTRODUCTION

Monocrotophos inhibits AChE enzyme resulting in accumulation of ACh at neuroeffector sites. Monocrotophos causes enhanced peristaltic movements in intestinal tissues by acting on muscarinic receptors. *Withania somnifera* is used as rejuvenative, adaptogenic, antistress and liver tonic since ayurvedic times (Mishra *et al.*, 2000). The present investigation was carried out to evaluate protective effect of *Withania somnifera* against monocrotophos–induced intestinal contractions.

MATERIALS AND METHODS

The effect of exogenously added acetylcholine in physiological salt solution was studied on the contractile behaviour of isolated duodenal smooth muscles of broiler chicks and the isometric responses were recorded using a 4 channel Polygraph (Biopac, USA). The objective of this study was to evaluate any modification in contractility of isolated duodenal tissue treated with monocrotophos alone or in combination with WRE.

The study was conducted in 5 groups with 12 birds in each group. Group I served as control. Monocrotophos (2 ppm in feed) was fed to chickens of group II to V and WRE (@ 100, 300 and 500mg/kg b. wt/day) was given in drinking water to birds of group III to V, respectively for 7 weeks.

Collection, Preparation and Mounting of Tissues

The chicks were sacrificed and duodenal portion of intestine was collected in a petridish containing aerated physiological saline solution (Krebs Henseleit solution, maintained at 25 ± 0.5^{0}C). Immediately, the tissues were placed on a filter paper moistened with physiological saline solution and cleaned by removing the connective tissue, pancreas and fat attached to them without causing damage to the smooth muscles. The tissues were mounted in an organ bath of 20 ml capacity containing Krebs solution (pH 7.4), which was continuously bubbled with atmospheric air and maintained at a temperature of 25 ± 0.5^{0}C. The tissues were allowed to equilibrate under a constant resting tension of 1 g for a period of 1 h. During equilibration period, the bathing fluid was regularly changed at every 15 min interval.

Calibration of Physiograph And Recording of Responses

Isometric contractions were recorded using a force transducer (0-50 g) connected to 4 channel polygraph (Biopac, USA).The scale of the tension was adjusted to 1 g. After equilibration period of 1 h, the drugs were added into the bath fluid and the isometric responses were recorded on 4 channel polygraph. To record the subsequent response(s) in the same tissue, sufficient rest to the tissue with repeated intervening washings was provided so that the tissue came to its base line after eliciting the previous response.

Only one set of experiment was carried out on each tissue to avoid the development of fatigue in the preparation and evade anomaly in the responses. After the experiment was over, all the tissues were taken out

of the bath and placed in a fold of filter paper to remove fluid and moisture and weighed immediately to get their weight. The absolute tension generated by the tissues was converted to the responses in g per g of tissue and per cent maximum response in terms of contraction.

Recording of normal contractility of isolated duodenum

Isolated duodenal tissue of chicks exhibited normal motility (peristaltic movements) in Krebs solution. After equilibration period of 1 h and before adding any drug, the normal motility was recorded for a period of 15 min.

Effect of KCL on isolated duodenum of chicks

After recording the normal motility of duodenum for 15 min, KCl (80 mM) was added to the tissue bath containing 20 ml of Krebs solution to record KCl induced responses.

A second response of KCl was recorded after providing sufficient rest of 10 min to the tissue with repeated intervening washings for at least 3 times so that the tissue came to its base line after eliciting the previous response.

Effect on acetylcholine-induced responses

Cumulative response of ACh was recorded after providing sufficient rest of 20 min to the tissue with repeated intervening washings for at least 5 times so that the tissue come to its base line after eliciting the previous response. Acetylcholine (0.01-3000 µM) was added to the organ bath in an increasing order to record the concentration-dependent responses. The tissues were allowed to show maximum response to each concentration of the agonist, which took about 1-1.5 min time. Thus, the cumulative concentration-response curves of acetylcholine were constructed.

All the contractile responses were calculated as g force per g of tissue. The cumulative concentration-contraction curves were constructed and compared between different groups and the maximum efficacy (E_{max}) of acetylcholine to find out shifting of the curves. The acetylcholine-induced contractions were converted to the per cent maximum responses. Thus, concentration-% maximum response curves were plotted and compared among different groups. On the basis of these curves, the median effective concentration (EC_{50})

Table 1:
Effect of cumulative concentrations of acetylcholine (Ach) on contraction (g/g of tissue) generated in isolated duodenum of chickens of different groups after 7 weeks of MCP (2 ppm in feed) treatment (mean ± S.E., n=6).

Group		Contraction (g/g of tissue)				
		I (Control)	II	III	IV	V
Extract/drug		-	MCP	MCP+WRE	MCP+WRE	MCP+WRE
WRE (mg/kg/day)		-	-	100	300	500
Acetylcholine (µM)	0.01	0.80±0.17	3.63±1.06	1.67±0.63	0.80±0.20	1.54±0.50
	0.03	1.72±0.56	5.19±1.45	3.29±0.71	1.41±0.42	2.48±0.33
	0.1	3.70±0.66	7.09±1.64	4.35±0.96	2.53±0.82	2.22±0.49
	0.3	6.94±1.45	9.67±1.26	6.22±0.63	4.01±0.72	6.38±0.85
	1	9.46±1.88	13.5±0.59	8.89±0.76	6.42±0.09	8.52±1.23
	3	11.6±1.24	15.7±0.76	11.3±1.12	7.48±1.13	11.08±1.55
	10	12.9±0.97	18.8±1.54	12.6±1.73	9.18±0.97	12.5±1.49
	30	13.4±1.10	18.8±1.54	14.05±1.54	10.3±0.80	13.2±1.56
	100	13.8±1.17	18.8±1.54	14.05±1.85	11.1±1.02	14.5±1.53
	300	13.8±1.17	18.8±1.54	14.05±1.85	11.1±1.02	14.5±1.26

Table 2:
Effect of cumulative concentrations of acetylcholine (Ach) on response generated in isolated duodenum of chickens of different groups after 7 weeks of MCP (2 ppm in feed) treatment (mean ± S.E., n=6).

Group		% maximum response				
		I (Control)	II	III	IV	V
Extract/drug		-	MCP	MCP+WRE	MCP+WRE	MCP+WRE
WRE (mg/kg/day)		-	-	100	300	500
Acetylcholine (µM)	0.01	11.2±3.12	18.3±1.72	11.7±2.99	6.97±1.51	8.37±2.33
	0.03	17.6±2.83	25.5±2.59	26.0±2.51	11.5±1.49	14.3±2.33
	0.1	30.3±2.26	52.6±1.29	35.6±2.48	20.0±2.99	21.8±2.20
	0.3	50.5±1.47	73.9±1.38	48.9±3.07	31.0±3.01	37.2±3.24
	1	68.5±2.05	85.2±2.39	65.3±3.29	49.0±2.99	50.0±2.67
	3	83.6±2.54	100.0±0.00	80.2±3.40	60.9±1.41	63.4±3.65
	10	94.3±3.22	100.0±0.00	100.0±0.00	77.5±3.18	71.6±2.39
	30	97.2±1.72	100.0±0.00	100.0±0.00	88.6±2.58	82.6±4.17
	100	100.0±0.00	100.0±0.00	100.0±0.00	100.0±0.00	85.6±3.22
	300	100.0±0.00	100.0±0.00	100.0±0.00	100.0±0.00	100.0±0.00

Table 3.
Effect of KCl (80mM) on contraction generated in isolated duodenum of chickens (% KCl response) of different groups after 7 weeks of MCP (2 ppm in feed) treatment (mean ± S.E., n=6).

Group		% KCl response				
		I (Control)	II	III	IV	V
Extract/drug		-	MCP	MCP+WRE	MCP+WRE	MCP+WRE
WRE (mg/kg/day)		-	-	100	300	500
Acetylcholine (µM)	0.01	6.11±1.16	18.1±2.99	14.3±2.17	6.96±1.92	7.65±2.27
	0.03	10.1±2.46	26.9±2.34	21.2±3.31	12.1±2.67	12.93±1.82
	0.1	23.1±1.27	44.1±3.20	27.6±1.47	21.1±2.78	19.7±2.77
	0.3	43.8±2.90	60.5±2.40	41.1±1.89	33.3±4.23	33.0±4.18
	1	59.1±2.23	84.3±3.71	61.6±2.11	54.35±3.53	45.5±7.20
	3	72.7±3.41	97.6±2.21	72.7±2.79	63.1±3.56	57.4±5.25
	10	81.8±2.74	108.3±3.51	82.7±2.10	78.3±3.71	64.7±4.86
	30	84.3±2.25	114.1±1.45	89.2±2.54	87.5±4.10	73.8±4.47
	100	87.1±3.12	114.1±1.45	89.2±2.54	93.3±3.60	82.3±5.02
	300	87.1±3.12	114.1±1.45	89.2±2.54	95.6±0.99	98.9±7.98

Table 4.
E_{max} and EC_{50} of acetylcholine (ACh) in duodenum of chickens of different groups after 7 weeks of MCP (2 ppm in feed) treatment (mean ± S.E., n=6).

Group	I (Control)	II	III	IV	V
Extract/drug	-	MCP	MCP+WRE	MCP+WRE	MCP+WRE
WRE (mg/kg/day)	-	-	100	300	500
E_{max}	13.8±1.17	18.8±1.54	14.05±1.85	11.1±1.02	14.5±1.26
EC_{50}	0.48±0.11	0.22±0.06	0.42±0.08	0.78±0.15	0.79±0.17

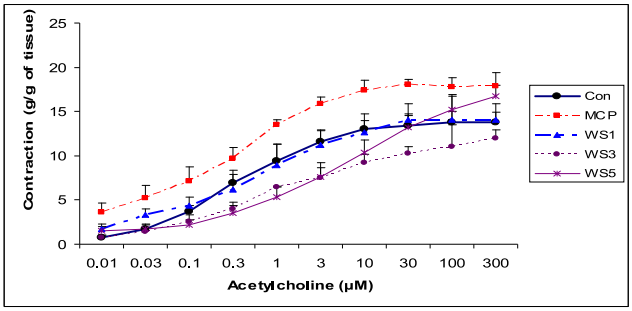

Fig.1:
Dose-response curves of acetylcholine (ACh) in isolated duodenal smooth muscles of chickens treated with MCP alone or in combination with WRE @ 100 (W_{100}), 300 (W_{300}) and 500 (W_{500}) mg/kg for 7 weeks (mean ± S.E, n = 6).

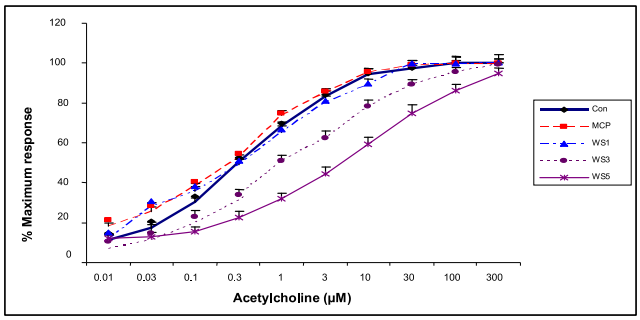

Fig.2:
Dose-% maximum response curves of acetylcholine (ACh) in isolated duodenal smooth muscles of chickens treated with MCP alone or in combination with WRE @ 100 (W_{100}), 300 (W_{300}) and 500 (W_{500}) mg/kg for 7 weeks (mean ± S.E, n = 6).

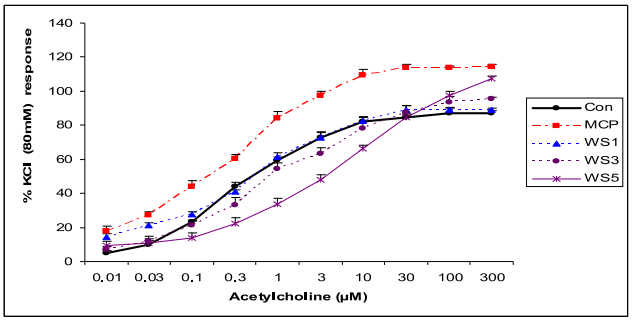

Fig.3:
Dose-% KCl response curves of acetylcholine (ACh) in isolated duodenal smooth muscles of chickens treated with MCP alone or in combination with WRE @ 100 (W_{100}), 300 (W_{300}) and 500 (W_{500}) mg/kg for 7 weeks (mean ± S.E, n = 6).

values were determined and compared among different groups for significant difference.

RESULTS AND DISCUSSION

The effect of cumulative doses of ACh (0.01, 0.03, 0.1, 0.3, 1, 3, 10, 30, 100 and 300µM) on isolated duodenal tissues of chicken treated with MCP alone and/or in combination with *W. somnifera* root extract for 7 weeks is presented in Table 1 & 2 and Fig. 1-4.

Acetylcholine, a cholinomimetic, produced dose dependent contraction of isolated duodenal smooth muscle in all groups of birds. In each dose of ACh the response of duodenum was significantly higher in MCP group in comparison to control. WRE reduced these responses significantly and dose dependently in

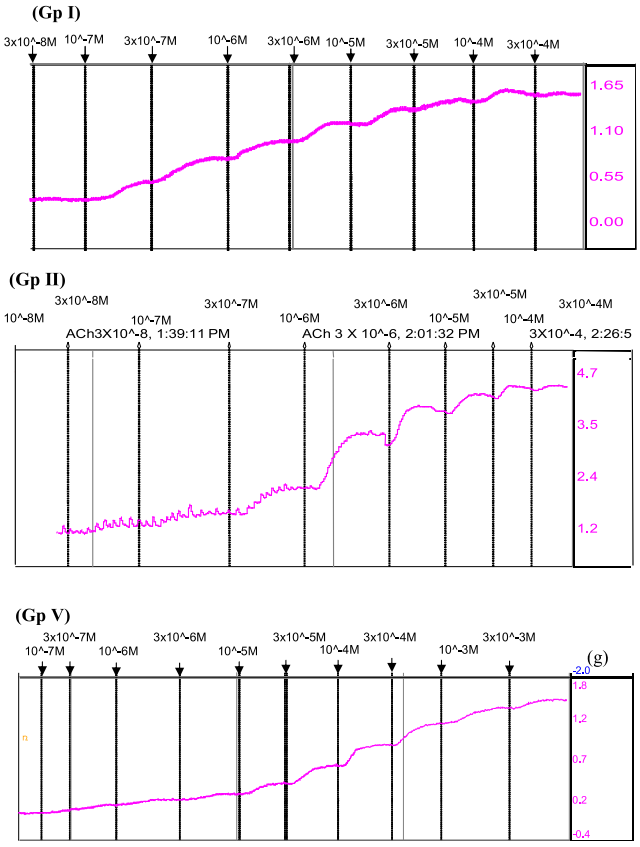

Fig.4:
Physiographic recordings of effect of cumulative doses of ACh on contractility of isolated poultry duodenum in birds of group I (Gp I), II (Gp II) and V (Gp V), respectively.

duodenal smooth muscle of MCP-treated birds. The E_{max} of ACh in control tissues were 13.8 ± 11.7 g/g of tissue. In MCP treated birds E_{max} of ACh was increased to 18.8 ± 1.54 g/g of tissue. WRE, at the dose rate of 300 mg/kg, significantly decreased the E_{max} to 11.1 ± 0.02 g/g of tissue. Also, WRE-treated birds of group III showed lowering in E_{max} (14.05 ± 1.85 g/g tissue contraction) when compared to MCP group II birds (Table 4). The dose-% maximum response curves reveal that the EC_{50} valves of ACh in MCP group was significantly reduced to 0.22 ± 0.06 µM in comparison to control (0.48 ± 0.11 µM). Dose- % maximum response curve of ACh in MCP group was shifted to the left in comparison to control. However, the curves of group III, IV and V were shifted to the right in comparison to MCP group.

As the response of ACh was recorded on duodenum of different groups of birds, there is a probability to encounter individual or group variability in the response of ACh.

To avoid the chance of this variation, initial 2 responses of KCl (80µM) where elicited in each tissues of different groups 15 min before recording the cumulative responses of ACh. On the basis of second KCl-induced contraction, the responses of ACh in tissues of different groups were calculated as percentage of KCl (80µM)-induced contraction and thus the dose-% KCl response curves of ACh have similarity with the dose response curves of ACh. Here also the dose-% KCl response curve of ACh of MCP group was shifted to the left with increase in E_{max}. In comparison to the curve of MCP group there was a shift to right in the curves of ACh in group III and V (Table 3).

DISCUSSION

ACh is a parasympathomimetic agonist, which binds with the muscarinic receptors of duodenal smooth muscle to induce contraction. It produced dose dependent contraction of the tissues in all groups. The E_{max} of ACh was significantly increased in MCP group in comparison to control. The EC_{50} valve of ACh was significantly reduced in MCP group and the dose-response curve of ACh of MCP group was shifted to the left. These findings indicated that the toxicant MCP has increased sensitivity of ACh in chicken duodenum. This increase in the responsiveness of ACh in MCP group may be because of increased binding of more number of ACh with its receptors and/or increase in the expression of the cholinergic muscarinic receptors. MCP is ACh inhibitor of this enzyme leads to accumulation of increased quantities of ACh at neuroeffector junctions. Hence, the probability of acceleration in binding of ACh with its receptors suits to be the proper explanation. Change in receptor population (Schrrurs *et al.,* 1980) and responsiveness of chicken duodenum to ACh and other agonists in some disease conditions have also been reported earlier by several workers (Veenedal *et al.,* 1985; Dina and Arowola, 1989; Jibike *et al.,* 1994). The toxic metabolites and by-products of gut microflorae were reported to alter gastrointestinal functions (Kenworthy, 1976; Dina and Arowdo, 1989). In present investigation aqueous extract of ashwagandha root significantly decreased the responses of ACh in duodenum of MCP-treated birds. The dose response curves were shifted dose dependently by WRE in these tissues. The EC_{50} valves of ACh where were also increased by WRE dose dependently in group III to V tissues. These findings demonstrated that ashwagandha extract reduced the responsiveness of muscarinic receptors of chicken duodenum to ACh, though the mechanism of action of WRE has not been elucidated, it may be hypothesized that the plant might have either decreased the expression of receptors or responsiveness of the tissues to acetylcholine. Contrary to our findings Ramarao and co-workers (1995) reported that glycowithanolide-sitoindosides VII to X and withaferin

A, isolated from roots of *W. somnifera* didn't influence the contractility of intestine in mice treated with morphine. The difference between the responses of present findings and that of Ramarao *et al.* (1995) is due to the fact that in their study morphine elicited inhibition of GIT transient. As the tissues were already relaxed in these case further spasmolytic effect of glycowithanolides probably could not be exhibited. On the other hand, in present findings MCP increased the sensitivity to ACh whereas the responsiveness of ACh could preferably be inhibited by WRE. Finally, the above findings suggest that WRE has protective efficacy against MCP-induced alterations in the responsiveness of cholinergic receptors to ACh in chicken duodenum.It is, thus, concluded from this investigation that ACh-mediated contractions on isolated duodenum of MCP-treated broilers became almost normal by the WRE.

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Research Article

EVALUATION OF LEMON JUICE AND GARLIC ON CLINICO-BIOCHEMICAL PROFILE AND ORGAN WEIGHTS OF BROILER CHICKEN

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ABSTRACT

The present study was planned to replace the antibiotics with Russian Penicillin (garlic) and lemon juice. Garlic have multi-folded pharmacological property *viz*. antibacterial, anti-oxidant, immuno-modulatory. However, lemon is vitamin-C enriched holding disinfectant action. Thirty-nine days of experimental period, zero day old 150 broiler chicks were divided into five groups and further each group divided into three replicate containing 10 chicks per replicate. Chicks of T-1, T-2, T-3, T-4 and T-5 groups were control, garlic (3% w/w), lemon (1% v/v), garlic plus lemon and sanitizer plus antibiotics groups, respectively. Garlic powder and lemon juice were mixed in feed and drinking water as feed additive and water sanitizer, respectively. Different biological parameters relating lipid profile, liver function, protein profile in serum and water consumption and body weight gain were analysed. A significant increase of water intake was found in garlic (T-2) and garlic plus lemon treated (T-4) broiler chicken compared to control. Similar increase in body weight gain as found in garlic plus lemon treated (T-4) broiler chicken compared to control. Serum glucose and SGOT level were significantly decreased in garlic plus lemon treated (T-4) broiler chicken compared to control. Interestingly the lemon juice significantly decreases the body weight gain of broiler chicken (T-3) compared to control. Hence, present study findings elucidating that the Broiler chicken can be reared without using antibiotics and water sanitizer replacing with easily available natural ingredient garlic and lemon.

Keywords: Broiler, garlic, lemon juice, biochemical parameters, carcass and body weight

INTRODUCTION

Feed additives are non-nutritive substance mixed with feed to increase its storage quality and also improve feed-intake, influencing the performance of animals (Tollba and Hassan, 2003). Garlic (*Allium sativum L.*) is a very commonly in use spice, possesses many medicinal properties with meagre side effect. Medicinal property of garlic is well explored and numerous scientific literatures disclose its medicinal activities such as antibacterial, anti-cancerous, antioxidant, anti-inflammatory, immuno-modulatory and hypoglycaemic activity (Reuter *et al.*, 1996). It also protects the cardiovascular system (Sivam, 2001). Its antimicrobial activity phonate the garlic nickname as 'Russian Penicillin'. It also contributes to replace the injudicious use of antibiotic to raise broiler chicken and helping to combat the emerging problem of current era 'antibiotic resistance' (Sanders and Sanders, 1992). Interestingly garlic is showing antibacterial activity against resistance bacteria (Jezowa *et al.*, 1966). Antimicrobial and growth promoting potency portraying the garlic as an antibiotic alternative for antibiotic free production of broiler chicken (Sivam, 2001). Water is a most important vital nutrient for health and participating in various physiological functions of body *i.e*. controlling body temperature, food digestion and absorption and waste elimination (Manz *et al.*, 2002). Citrus fruits are the important fruit tree crop in the world and lemon the third important amongst the Citrus species (González-Molina *et al.*, 2010). It contains several bioactive components namely citric acid, polyphenol, ascorbic acid, minerals and also possessing commercial value for food industry (González-Molina *et al.*, 2010). Lemon juice is vitamin-C enriched and acts as a disinfectant (D'Aquino and Teves, 1994). Lemon juice is major health concern and participating in reducing the weight (Marti *et al.*, 2009; Assini *et al.*, 2013). Various studies suggested that daily intake of lemon showing good effect on health and have ingredients for lipid metabolism and obesity (Miyake *et al.*, 2006). In present study garlic powder was added to broiler feed as feed additive and lemon juice pour in drinking water in place of water sanitizer to curtail the use of antibiotics for broiler production.

MATERIALS AND METHODS

Experimental Design

Broiler chicken of Cobb strain was purchased from Sunrise Hatcheries, UPSIC Industries State, Chandauli, (UP), India. The broilers chicks were kept in faculty poultry farm for 39 days of experimental period

under standard management condition. A total of 150 broiler chicks were randomly divided into five groups with thirty chicks in each group. Thirty chicks of each group were further sub-divided into three replicate containing ten chicks in each replicate. The groups were named T-1 (control group), T-2 (garlic treated group), T-3 (lemon treated group), T-4 (garlic plus lemon treated group) and T-5 (sanitizer plus antibiotics group). The broiler chicken of control group (T-1) was without any treatment, whereas T-2, T-3, T-4 and T-5 treated with garlic powder as feed-additive (3% w/w), lemon juice mixed in drinking water as sanitizer (1% v/v), garlic plus lemon treated and water sanitizer "Elimin-8" (0.5ml per litre of drinking water) plus occasionally antibiotics treatment, respectively. The commercially available feed was used for rearing the broiler chicks. Prior to start the experiment received permission from Institutional Animal Ethics Committee and study was conducted strictly in accordance to Ethical Committee guidelines.

Garlic and lemon preparation

The garlic powder was purchased from local market. Garlic powder was used as feed supplement and added directly in poultry feed at the rate of 3g per 100g of poultry feed (3% w/w). However, lemon juice was used as water disinfectant. Lemon was purchased from local market and juice has squeezed out. Lemon juice obtained was mixed in drinking water at the rate of 1ml per 100ml in drinking water (1%w/v).

Sampling and analysis

Blood samples were collected on day 39th *i.e.* the day broiler chicken sacrificed and weight of different part of carcass had performed. The collected blood samples (5 ml) were transferred to clean, dry and sterilized tube and kept in slanted position inside the freeze for about half an hour. The blood samples stored in freeze were centrifuged at 400*g* for 20 min and serum obtained had kept at × 60°C pending analysis. The stored serum samples were used for the analysis of different parameters namely glucose, protein profile (albumin, bilirubin total & total protein), kidney function parameter (uric acid), lipid profile (triglyceride, cholesterol), and liver function parameters (Alkaline phosphatase, SGPT, SGOT) using automated Random Access Clinical Chemistry Analyzer with system pack (EM-200, ERBA diagnostic Mannheim, GmbH, Germany).

Statistical analysis

Data were analyzed using statistical software SPSS version 20 and presented as mean ± standard deviation. The difference between the groups were analysed by one-way analysis of variance (ANOVA) at $p<0.05$ level of significance for differences in mean under Duncan's Multiple Range Test.

RESULTS

Result of garlic powder and lemon juice on clinical and biochemical profile

It was found that the bedding material of the broiler chicken of lemon treated group had more wet than the bedding material of all other experimental groups. Table.1, showing a significant increase in water intake was found in broiler chicken of garlic (11.70 ±2.12 L) and garlic plus lemon (11.49±1.94 L) treated groups as compared to control (9.19±2.14 L).

As shown in Fig.1, a significant decrease in serum glucose level was found in broiler chicken of garlic plus lemon treated group (273.07±55.97 mg/dl) as compared to control group (329.12±30.17 mg/dl). Also a non-significant decrease in serum glucose level was noted in garlic, lemon and sanitizer treated group compared to control group. Fat biomarker, serum triglyceride level was decreased non-significantly in garlic plus lemon treated group as compared to control, garlic, lemon and sanitizer treated groups. A significant decrease in serum cholesterol level was found in garlic plus lemon (114.33±25.36 mg/dl) treated group as compared to garlic (150.22±31.23 mg/dl), lemon (142.89±24.06 mg/dl) and sanitizer (146.56±27.06 mg/dl) treated groups. However, the decrease in serum cholesterol was non-significant in garlic plus lemon treated group compared to control group.

A non-significant decrease in serum SGPT level was found in lemon and garlic plus lemon treated groups compared to control, garlic and sanitizer treated groups. However, a significant decrease in serum SGOT level was found in garlic plus lemon (219.09±51.83 U/Lit.) treated group as compared to control (271.51±54.10 U/Lit.), garlic (280.42±48.39 U/Lit.), lemon (302.83±51.15

Table.1:
Effect of Garlic powder and lemon juice on water intake (Lit.) and uric acid (mg/dl) in serum sample of broiler chickens.

Water intake from Day 23rd to 39th (Lit.)					
Groups (n=50)	T-1 (Control)	T-2 (Garlic)	T-3 (Lemon)	T-4 (Garlic+Lemon)	T-5 (Sanitizer)
Water intake (lit.)	9.19 ± 2.14 ^{a}	11.70 ± 2.12 ^{c}	9.01 ± 2.79 ^{a}	11.49 ± 1.94 $^{b, c}$	10.06 ± 2.01 $^{a, b}$
Uric acid level in serum on day 40th (mg/dl)					
Groups	T-1 (Control)	T-2 (Garlic)	T-3 (Lemon)	T-4 (Garlic+Lemon)	T-5 (Sanitizer)
Uric Acid (mg/dl)	4.02± 1.24 $^{a, b}$(n=9)	4.58 ± 1.26 ^{b}(n=9)	3.76 ± 1.33 $^{a, b}$(n=8)	3.25 ± 0.63 ^{a}(n=8)	3.55 ± 1.12 $^{a, b}$(n=8)

All values are in (Mean ± S.D.). The level of significance between the groups were analysed at $p<0.05$ using one way ANOVA-Duncan. Same superscripts between the groups indicate no-significant difference.

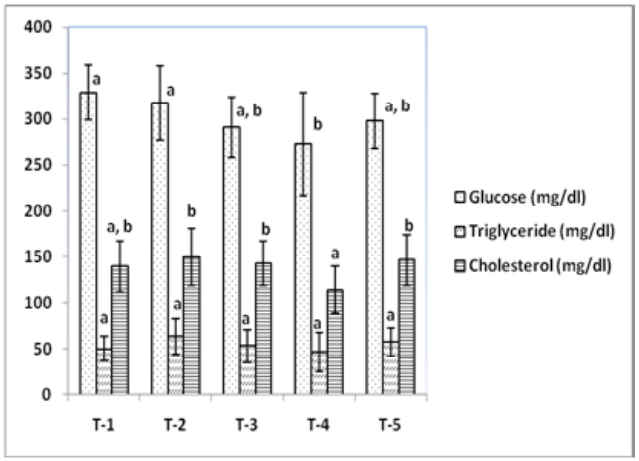


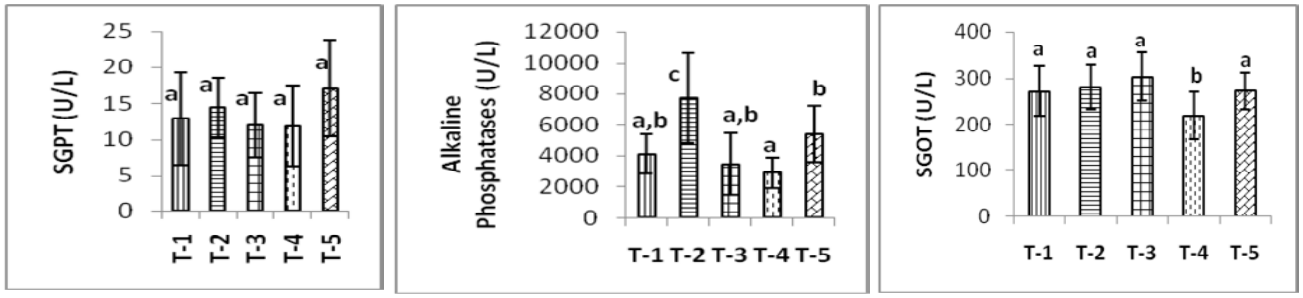
Fig.1:
Effect of on glucose, triglyceride and cholesterol level (mg/dl) in serum sample

U/Lit.) and sanitizer (272.41±39.70 U/Lit.) treated groups. A significant decrease in serum alkaline phosphatases level was found in garlic plus lemon (2910.25±989.66 U/Lit.) treated group as compared to garlic (7736.13±2898.00 U/Lit.) treated group. However, a non-significant decrease in serum alkaline phosphatase level was found in lemon and garlic plus lemon treated groups as compared to control (Fig.2).

The kidney function biomarkers serum creatinine (mg/dl) was found beyound detectable limit. However, in garlic plus lemon treated group none of the sample was found creatinine positive. A non-significant decrease in serum uric acid level was observed in broiler chicken of lemon, garlic plus lemon and sanitizer treated groups as compared to control(Table 1).

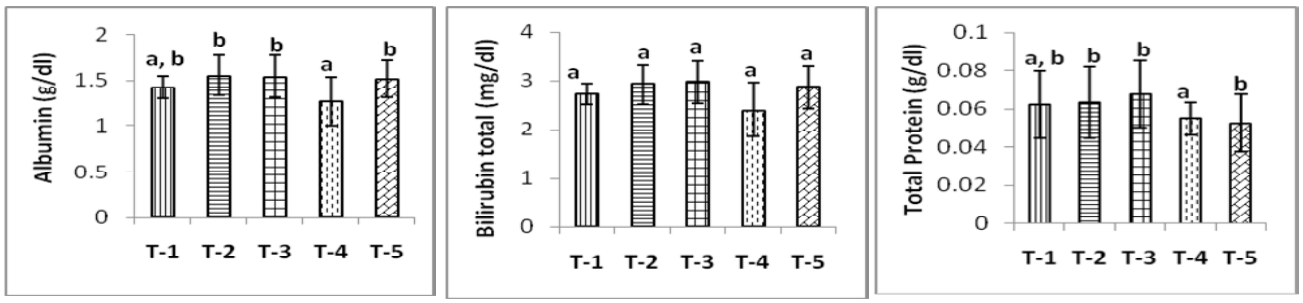
As depicted in Fig.3, a non-significant increase in serum albumin level was found in garlic, lemon and sanitizer treated groups as compared to control. However, a significant decrease in serum albumin level was noted in garlic plus lemon treated group as compared to garlic, lemon, and sanitizer treated groups. Serum bilirubin total level was found a non-significant decrease in garlic plus lemon and sanitizer treated groups as compared to control, garlic and lemon treated groups. A non-significant decrease in serum total protein level was found in chicken of garlic plus lemon treated group as compared to control showing similar pattern in decrease of serum albumin and bilirubin total level. However, a significant decrease in serum total protein level was found in garlic plus lemon (2.40±0.56g/dl) group as compared to garlic (2.93±0.40g/dl), lemon (2.98±0.43g/dl), and sanitizer (2.87±0.44g/dl) treated groups.

Fig.4 shows the effect of Garlic powder and lemon juice on body weight gain on day 20th and 30th of treatment period. A significant increase in body weight gain of the broiler chicken was found in garlic and garlic plus lemon treated group as compared to control,



A. B. C.

Fig.2:
Effect on (A). SGPT, (B). SGOT and (C). Alkaline phosphatises (U/lit.) in serum samples



A. B. C.

Fig.3:
Effect on (A). Albumin (g/dl), (B). Bilirubin total (mg/dl) and (C). Total protein (g/dl) in serum sample

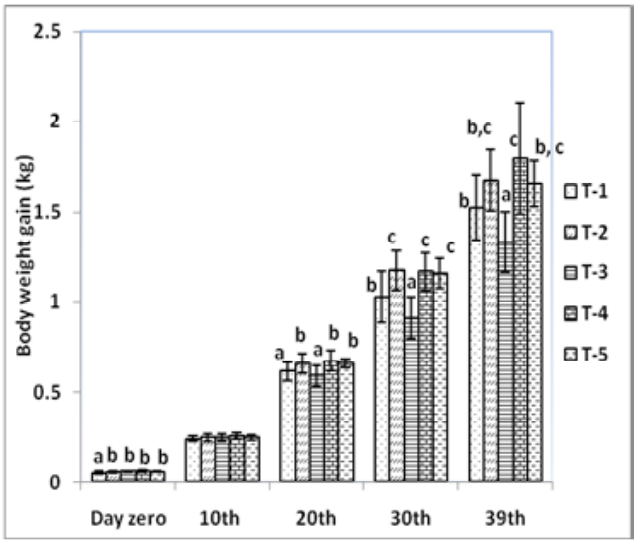


Fig.4:
Effect on body weight gain (kg) at different time intervals

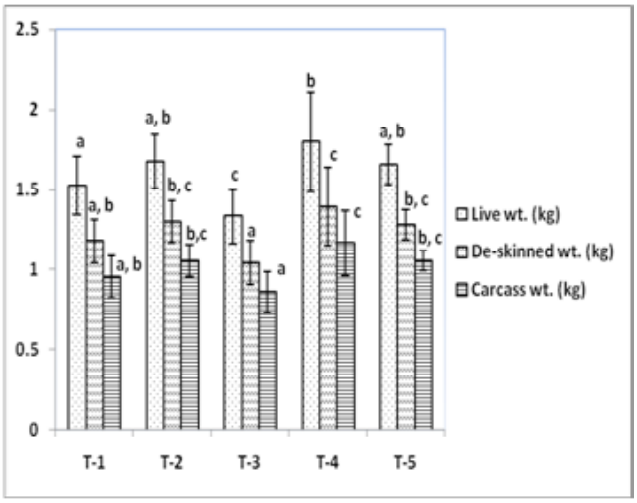


Fig.5:
Effect on live weight (kg), De-skinned weight (kg) and carcass weight (kg)

respectively. Similarly on day 20th and 30th, a significant increase in body weight gain was found in sanitizer treated group as compared to control group, respectively. Also on day 39th of treatment period, a significant and non-significant increase in body weight gain was found in garlic plus lemon treated and garlic treated groups as compared to control, respectively. In contrary, on day 30th and 39th, a significant decrease in body weight gain was found in broiler chicken of lemon treated groups compared to control, garlic, garlic plus lemon and sanitizer treated groups.

Effect of Garlic powder and lemon juice on live, de-skinned, organs and carcass weights

As depicted in Fig.5, a significant increase in live weight gain of broiler chicken of garlic plus lemon

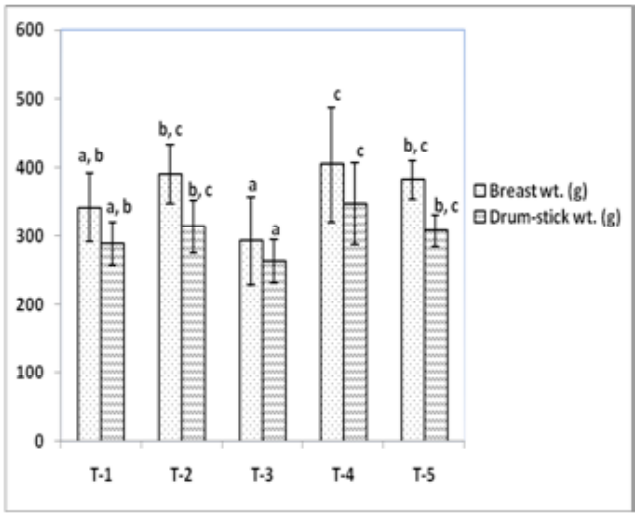


Fig.6:
Effect on weights (g) breast and drum-stick weight

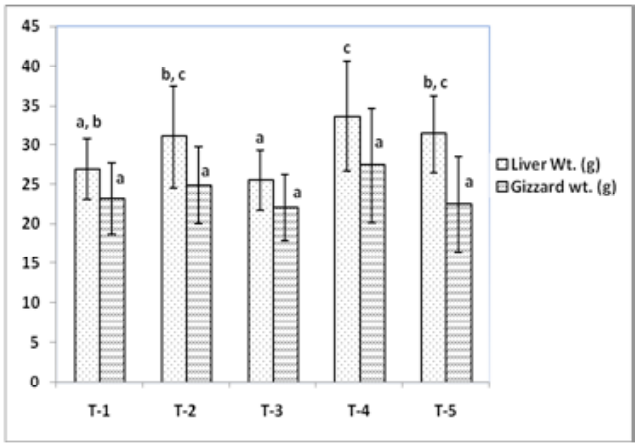
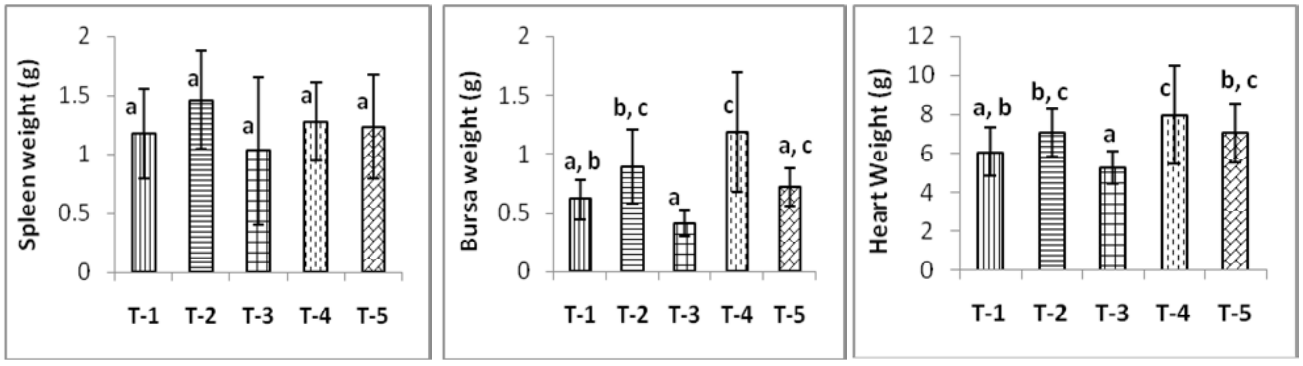


Fig.7:
Effect on liver and gizzard weight (g)

(1.80±0.31kg) treated group was found as compared to control (1.52±0.18kg). However, live weight gain of chicken of garlic and sanitizer treated group was a non-significant increase as compared to control. In contrary broiler chicken of lemon (1.33±0.17kg) treated group, a significant decrease in live weight gain was found as compared to control (1.52±0.18kg), garlic (1.68±0.17kg), garlic plus lemon (1.80±0.31kg) and sanitizer (1.66±0.13kg) treated groups.

Similar pattern of changes found in both de-skinned and carcass body weight gain *i.e.* a significant increase of de-skinned body weight and carcass weight gain were found in chiken of garlic plus lemon treated group as compared to control, respectively. In contrary, a non-significant decrease in de-skinned and carcass weight gain were found in lemon treated group as compared to control, respectively and interestingly sinificant with garlic, garlic plus lemon and sanitizer treated groups.



A. B. C.

Fig.8:
Effect on (A). Spleen weight (g) B. Bursa weight (g) and C. heart weight (g)
Figures 1-8 shows effect of Garlic powder and lemon juice on various parameters of broiler chickens (n==9).
All values plotted in the figures are in (Mean ± S.D.). The level of significance between the groups were analysed at $p<0.05$ using one way ANOVA-Duncan. Same superscripts between the groups indicate no-significant difference.

Fig.6, shows a similar pattern of change in both breast and drum-stick weight gain *i.e.* significant increase of breast and drum stick weight of broiler chicken in garlic plus lemon treated (404.44±83.94g; 347.22±59.64g) group was found as compared to control, respectively. In contrary, broiler chicken of lemon treated group, a non-significant decrease of breast and drum-stick weight gain was found as compared to control, however significant change with garlic, garlic plus lemon and sanitizer treated groups, respectively.

A significant increase of liver weight gain was found in chicken of garlic plus lemon (33.65±7.27g) treated group as compared to control (26.87±4.50g) and lemon (25.48±4.14g) treated groups. However, no significant change was found in gizzard weight (Fig.7)

A non-significant change of spleen weight gain was found between the different experimental groups, The bursal weight of broiler chicken of garlic plus lemon (1.18±0.51g) treated group was significantly increased as compare to control (0.62±0.17g). Similar pattern of changes in bursa and heart weight gain noted *i.e.* a significant increase in bursa and heart weight gain were found in broiler chicken of garlic plus lemon (1.18±0.51g; 7.94±2.51g) treated groups as compared to control (0.62±0.17g; 6.09±1.22g) Fig.8).

DISCUSSION

Interestingly, a significant increase in water intake was noted in both garlic and garlic plus lemon treated groups as compared to control group in growing phase of production cycle *viz.* from day 23rd to 39th. There are reports about the effect of garlic treatment over body weight gain, however literature has not found in correlation with influence of garlic on water intake of broiler chicken. A significant decrease in serum glucose level was found in garlic plus lemon treated group compared to control group. Also an appreciable decrease in serum glucose level was found in lemon treated group as compared control. The finding of present study indicating that garlic plus lemon juice treatment helps in reducing the serum glucose level. Similar decrease in glucose level was also reported in garlic treated broiler (Ashour, 2002; Fallah, 2014; Hossain *et al.*, 2014). Also garlic treatment decreases the glucose level in diabetic rabbit (Sher *et al.*, 2012). The potency of garlic to decrease the serum glucose level is beneficial in both hypo and hyperglycaemic condition (Hossain *et al.*, 2014).

Lipid profile parameters *i.e.* triglyceride and cholesterol level were decreased appreciable in serum of garlic plus lemon treated group compared to control. Similar decrease of both the parameters was also reported in garlic treated broiler (Issa and Omar, 2012; Fallah, 2014). Lemon juice is also participating in reducing the triglycerides (Behboudi *et al.*, 2016). The possible mechanism of garlic in lowering the cholesterol is garlic constituent 'Allicin' in-combination with -SH group of Acetyl-CoA that participates in cholesterol biosynthesis or organosulphur compounds helping to lower the cholesterol (Mathew *et al.*, 2004; Puvaca *et al.*, 2014). The liver X receptor-α (LXRα) regulates cholesterol, triglyceride and glucose homeostasis and garlic treatment lower down the expression of receptor thus regulating homeostasis of cholesterol, triglyceride and glucose (Mohammadi and Oshaqhi, 2014).

There was a significant decrease in serum SGOT activity found in garlic plus lemon treated group compared to control group. However, a non-significant change of serum SGPT level was noted between the groups. Similar decrease in SGOT activity was reported in turkey hens and broiler chicken treated with garlic. Garlic is showing hepato-protective activity by inhibiting

the protein catabolism or promoting antioxidant activity (Ajayi *et al.*, 2009; Jimoh *et al.*, 2012; Krauze *et al.*, 2012). Similarly garlic plus lemon treatment non-significantly decreases the serum alkaline Phosphatase level as compared to control. The Ajayi *et al.* (2009) also found similar decrease in ALP in garlic treated broiler chicken.

There was a significant decrease in uric acid level found in garlic plus lemon treated group compared to garlic treated group indicating garlic plus lemon treatment have the potency to reduce the serum urea level. There are many reports showing garlic alone is significantly influencing the uric acid level (Krauze *et al.*, 2012; Hossain *et al.*, 2014).

A non-significant increase of non-enzymatic antioxidants *viz.* albumin and bilirubin were found in garlic treated group compared to control group. Krauze *et al.* (2012) reported similar increase of albumin and bilirubin level helps to protect from free radicals and oxidative stress. However, significant decrease of serum albumin and total protein levels was found in garlic plus lemon treated group compared to garlic treated group indicating garlic plus lemon is significantly influencing the serum protein level.

Present study discloses that garlic and lemon in-combination significantly ameliorate the body weight gain through improving gut environment. However, lemon juice treatment alone significantly decreases the body weight gain on day 30^{th} and 39^{th}. Scientific literatures are exploring the multifactorial pharmacological efficacy of garlic (antibacterial, antioxidant) and therefore, garlic augumenting the growth of broiler (Akhtar *et al.*, 1984; Agarwal, 1996; Nogueira *et al.*, 2003). However, report about the effect of lemon juice over the growth of broiler chicken is lacking and its anti-obesity potency reported in other species (Ross *et al.*, 2001; Ezz *et al.*, 2016). The main finding of the present study are as follows (i). without antibiotics, rearing of broiler chicken can be performed, (ii). lemon juice as water disinfect (1% v/v) hamper the body-weight gain of the broiler chicken, (iii). garlic powder (3%w/w) as feed additive plus lemon juice as water disinfectant (1%v/v) giving interesting result *viz.* increasing body-weight gain and water intake both in growing phase of broiler chicken, and reducing serum glucose and SGOT level.

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Research Article

IN VITRO ANTIBACTERIAL ACTIVITY OF ENDOPHYTIC BACTERIA ISOLATED FROM *MORINGA OLEIFERA* LEAVES

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ABSTRACT

The present study was conducted to isolate and identify endophytic bacteria from leaves of *Moringa oleifera* (Munga) and *in vitro* antibacterial activity was observed on Gram positive and Gram negative bacteria. Twenty leaves samples from *Moringa oleifera* were taken. The leaves were sterilized and incubated into King's B agar medium and then again sub-cultured into blood agar and then transferred into BHI broth. The morphological and biochemical characteristics of endophytic bacteria isolated from *Moringa oleifera* were studied. Antibacterial activity was studied by the disc diffusion method with known antibiotic ciprofloxacin (CIP) as standard. Twenty bacterial isolates from leaves of *Moringa oleifera* were isolated. The microscopic examination of endophytic bacteria showed that isolates from leaves of *Moringa oleifera* were gram negative rods. The biochemical characterization of endophytic bacterial isolates from *Moringa oleifera* showed positive reaction to catalase and oxidase, and negative reaction to coagulase test. All the isolates had shown negative reaction to various enzymic activity tests. Endophytic bacteria from leaves of *Moringa oleifera* had shown antibacterial activity against *Staphylococcus aureus* and *Salmonella* Typhimurium.

Key words: Antibacterial, Ciprofloxacin, Endophytes, *Moringa oleifera.*

Introduction

Global health problems due to drug resistance among pathogenic bacteria, inefficacy of current antibacterial agents to several bacterial infections and appearance of life-threatening viruses have necessitated the urgent needs for new and effective antimicrobial agents. In recent years, search for new therapeutic agents have been directed towards endophytes (Willson, 1995). The term endophyte (Gr. *endon*, within; *phyton*, plant) was first coined in 1866 by De Bary. An endophyte can be defined as a microorganism such as fungi or bacteria that expends either the complete or part of its lifecycle within the healthy tissues of a living plant, typically causing no symptoms of disease (Anjum and Chandra, 2015). The relationship that they establish with the plant varies from symbiotic to pathogenic. Literature shows that some bacteria which live attached to plant have ability to promote plant growth (Compant *et al.*, 2010 and Beneduzi *et al.*, 2013).

Endophytic bacteria seem to be distributed in most plant species and have been isolated from roots, leaves and stems, and a few from flowers, fruits, and seeds (Lodewyckx *et al.*, 2002). Endophytes existing in plants have a wide range of antimicrobial strains, which are the important potential sources of antimicrobial substances. Some endophytes could excrete antimicrobial compounds that may be involved in a symbiotic association with a host plant. Therefore, there is a huge potential to screen novel, highly active and low toxicity antimicrobial substances from endophytes (Strobel and Daisy, 2003).

The objective of the present study was to isolate endophytic bacteria from Moringa Oleifera (munga) leaves, their identification and investigation of their antibacterial activity against three gram positive bacteria viz. *Staphylococcus aureus*, *Streptococcus pyogens* and *Bacillus cereus* and gram negative bacteria *Escherichia coli*, *Salmonella* Typhimurium and *Klebsiella pneumoniae*.

MATERIALS AND MATHODS

Collection of Samples

Fresh leaves of *Moringa oleifera* (Munga) were procured from Department of Botany, J.N.K.V.V. and N.D.V.S.U campus, Jabalpur. Mature healthy plant leaves were collected from two different places of Jabalpur. Ten samples from each area were taken and processed for further isolation of endophytic bacteria. Samples were immediately brought to laboratory and were used within 24 hrs and finally processed for isolation of endophytic bacteria.

Sterilization of leaves and sterility check

The sterilization of leaves and isolation of endophytic bacteria from the leaves was done according to Mahajan *et al.* (2014), with some modifications (Fig.1,2 & 3).

To confirm that the surface of leaves were effectively sterilized, 1 ml of the sterile distilled water that was used in final rinse of surface sterilization procedures were plated on to nutrient agar media and

incubated at 37°C for 24 hrs. Bacterial growth was observed after 24 hrs. Growth of bacteria on the nutrient agar media showed that the leaves were not effectively sterilized.

Preparation and sterilization of media

King's B (KB) media (HiMedia), Mueller Hinton media (HiMedia), Blood agar media (HiMedia) and BHI broth (HiMedia) were prepared by adding agar into the distilled water. Hot plate was used for the proper mixing of media and autoclaved at 121°C for 15-20 minutes at 15 lbs.

Inoculation of leaves and isolation of endophytic bacteria

The media was poured into different autoclaved Petri plates and leaves of the plants were embedded in Petri plates. These plates were then incubated at 37°C for 24 hrs. Characterization of the bacteria was done according to its morphology and by Gram's staining. After that a single colony was transferred into BHI broth and incubated at 37°C for 24 hours (Fig. 4 & 5).

Purification of endophytic bacteria

For purification of endophytic bacteria, subculturing was mainly done by streaking a loop full of BHI broth on the fresh pre solidified blood agar plates and then incubated at 37°C for 24 hrs. After incubation the colony was transferred into BHI broth and then incubated at 37°C for 24 hrs and purity was checked by Gram's staining and stored for further work (Fig. 6).

Table 1:
List of procured culture of bacteria

S. No.	Bacteria	ATCC Catalogue No.
1.	*Escherichia coli*	25922
2.	*Klebsiella pneumonia*	700603
3.	*Salmonella* Typhimurium	13311
4.	*Bacillus cereus*	11778
5.	*Staphylococcus aureus*	6538
6.	*Streptococcus pyogenes*	12386

Antibacterial activity of endophytic bacteria *In vitro* study

Preparation of inoculums of known culture

Mc-Farlands standard was used for the determination of concentration of known culture as described by Henric *et al*. (1956). 1 ml of known culture containing 3.0×10^9 cfu/ml was used for antibacterial activity of endophytic bacteria (Table 1).

Assay of antibacterial activity

For determination of antibacterial activity of endophytic bacteria preparation of antibacterial disc was done according to Kirubaharan *et al*. (1999) with slight modifications.The prepared bacterial inoculums were evenly spread on a sterile Mueller Hinton agar plate as per method described by Bauer *et al.* (1969). The known antibiotic Ciprofloxacin (CIP) disc was simultaneously placed as a control for antibiotic sensitivity. The dried disc was incubated at 37°C for 24 hrs. Result was recorded as positive (growth) or negative (no growth) and zone of inhibition of growth exerted by these impregnated discs.

RESULTS AND DISCUSSION

Total twenty strains of endophytic bacteria were isolated from leaves of *Moringa oleifera*, ten strains from each place *viz.* Department of Botany, J.N.K.V.V. and N.D.V.S.U. campus, were obtained and identified by morphological, biochemical and molecular methods. *In vitro* antibacterial activity was evaluated against six known pathogenic bacteria. Knowledge on the diversity of endophytic bacteria is important for both ecological and biotechnological studies.

Growth characteristics of endophytic bacteria isolated from *Moringa oleifera* from Department of Botany, J.N.K.V.V. and N.D.V.S.U. campus in King's B media showed that 85 per cent were circular in shape while 15 per cent were irregular, 55 per cent colonies had raised elevation, 35 per cent had convex elevation, while 10 per cent were flat on petri plate. Margin of 85 per cent colonies were entire while 15 per cent were filamentous, the surface of the growth was smooth for 80 per cent of colonies and 40 per cent growth were opaque and white in colour while 60 per cent were translucent (Table 2).

The endophytic bacterial colonies grown on King's B agar were transferred to blood agar plates and incubated at 37°C for 24 hrs. The growth of endophytic bacteria from the leaves of *Moringa oleifera* were observed. All the isolates from leaves of *Moringa oleifera* were non-haemolytic in nature.

Colonies of endophytic bacteria which were grown on blood agar were transferred to the sterile BHI broth tubes and incubated at 37°C for 24 hrs. The growth of endophytic bacteria from leaves of *Moringa oleifera* were observed.

Endophytic bacteria from *Moringa oleifera* leaves collected from Department of Botany, J.N.K.V.V. and N.D.V.S.U. campus in BHI broth showed characteristics as, 60 per cent isolates showed surface growth and 100 per cent isolates showed turbidity. Sediment formation was seen in 85 per cent isolates and 45 per cent isolates showed odour formation (Table 3).

The microscopic examination of endophytic bacterial isolates had shown that all endophytic bacterial isolates from leaves of *Moringa oleifera* were Gram negative rods (Table 4). Sunkar and Nachiyar, (2013) found that 95 per cent of endophytic bacteria isolated from *Brassica oleracea* were Gram negative rod. Baghat *et al*. (2014) found that 90 per cent of isolated endophytic bacteria from *Capparis sinaica* were Gram positive in nature.

The endophytic bacteria isolated from *Moringa*

Table 2:
Growth of endophytic bacteria isolated from *Moringa oleifera* leaves on King's B media

S. No.	Isolate No.	Form	Elevation	Margin	Surface	Opacity	Chromogenesis
1.	JM-1a	Circular	Convex	Entire	Smooth	Opaque	Absent
2.	JM-1b	Irregular	Flat	Filamentous	Rough	Translucent	Absent
3.	JM-1c	Circular	Raised	Entire	Smooth	Opaque	Absent
4.	JM-1d	Circular	Raised	Entire	Smooth	Translucent	Absent
5.	JM-1e	Circular	Raised	Entire	Smooth	Opaque	Absent
6.	JM-2a	Circular	Convex	Entire	Smooth	Opaque	Absent
7.	JM-2b	Irregular	Raised	Filamentous	Rough	Opaque	Absent
8.	JM-2c	Circular	Convex	Entire	Smooth	Translucent	Absent
9.	JM-2d	Circular	Raised	Entire	Smooth	Translucent	Absent
10.	JM-2e	Circular	Convex	Entire	Smooth	Opaque	Absent
11.	NM-1a	Circular	Convex	Entire	Smooth	Translucent	Absent
12.	NM-1b	Irregular	Flat	Filamentous	Rough	Translucent	Absent
13.	NM-1c	Circular	Raised	Entire	Ringed	Translucent	Absent
14.	NM-1d	Circular	Raised	Entire	Smooth	Translucent	Absent
15.	NM-1e	Circular	Convex	Entire	Smooth	Opaque	Absent
16.	NM-2a	Circular	Convex	Entire	Smooth	Translucent	Absent
17.	NM-2b	Circular	Raised	Entire	Smooth	Translucent	Absent
18.	NM-2c	Circular	Raised	Entire	Smooth	Opaque	Absent
19.	NM-2d	Circular	Raised	Entire	Smooth	Translucent	Absent
20.	NM-2e	Circular	Raised	Entire	Smooth	Translucent	Absent

Table 3:
Growth of endophytic bacteria isolated from *Moringa oleifera* leaves in BHI broth

S. No.	Isolate number	Surface growth	Turbidity	Sediment	Odour	Pigmentation
1.	JM-1a	Absent	Present	Present	Present	Absent
2.	JM-1b	Present	Present	Absent	Present	Absent
3.	JM-1c	Absent	Present	Present	Absent	Absent
4.	JM-1d	Present	Present	Absent	Absent	Absent
5.	JM-1e	Present	Present	Present	Absent	Absent
6.	JM-2a	Absent	Present	Present	Present	Absent
7.	JM-2b	Present	Present	Present	Present	Absent
8.	JM-2c	Absent	Present	Present	Absent	Absent
9.	JM-2d	Present	Present	Present	Absent	Absent
10.	JM-2e	Absent	Present	Present	Present	Absent
11.	NM-1a	Present	Present	Present	Absent	Absent
12.	NM-1b	Present	Present	Present	Absent	Absent
13.	NM-1c	Present	Present	Present	Present	Absent
14.	NM-1d	Absent	Present	Absent	Absent	Absent
15.	NM-1e	Present	Present	Present	Present	Absent
16.	NM-2a	Present	Present	Present	Present	Absent
17.	NM-2b	Absent	Present	Present	Absent	Absent
18.	NM-2c	Present	Present	Present	Absent	Absent
19.	NM-2d	Absent	Present	Present	Present	Absent
20.	NM-2e	Present	Present	Present	Absent	Absent

oleifera of Department of Botany, J.N.K.V.V. and N.D.V.S.U. campus had shown positive reaction to catalase, oxidase and ONPG, and negative reaction to Coagulase, VP, Urease, Arginine utilization and Sugar fermentation test (Table 5).

In the enzymic activity test cellulolytic, amylolytic and esterolytic activity was not observed with endophytic bacteria isolated from *Moringa oleifera* (Table 06). Soman (2018) found that endophytic bacteria isolated from different varieties of babool leaves did not show any enzymic activity reaction viz. cellulase, amylase and protease activity test reaction. Khanam and Chandra (2015) conducted a study in which the isolates from the dye yielding plant *Beta vulgaris* did not show any enzymic activity reaction. In contrast, El-Deeb *et al.* (2013) observed that endophytic bacteria isolated from *Plectranthus tenuiflorus* had exhibited extracellular enzymatic activity.

The antibacterial activity of endophytic bacteria was evaluated against various Gram positive and Gram negative pathogenic bacteria namely *Bacillus cereus, Staphylococcus aureus, Streptococcus pyogenes, Escherichia coli, Klebsiella pneumoniae* and *Salmonella* Typhimurium. Results were recorded for the formation of zone of inhibition around the disc. The inhibitory zone around the disc indicated absence of bacterial growth reported as sensitive and absence of zone reported as resistant. The *in vitro* antibacterial activity of the

Table 4:
Gram's staining of endophytic bacteria isolated from *Moringa oleifera* leaves

S. No.	Isolate number	Gram's staining	Shape	Types of bacteria
1.	JM-1a	Negative	Rod	1
2.	JM-1b	Negative	Rod	1
3.	JM-1c	Negative	Rod	1
4.	JM-1d	Negative	Rod	1
5.	JM-1e	Negative	Rod	1
6.	JM-2a	Negative	Rod	1
7.	JM-2b	Negative	Rod	1
8.	JM-2c	Negative	Rod	1
9.	JM-2d	Negative	Rod	1
10.	JM-2e	Negative	Rod	1
11.	NM-1a	Negative	Rod	1
12.	NM-1b	Negative	Rod	1
13.	NM-1c	Negative	Rod	1
14.	NM-1d	Negative	Rod	1
15.	NM-1e	Negative	Rod	1
16.	NM-2a	Negative	Rod	1
17.	NM-2b	Negative	Rod	1
18.	NM-2c	Negative	Rod	1
19.	NM-2d	Negative	Rod	1
20.	NM-2e	Negative	Rod	1

Table 6:
Enzymic activity test reaction of endophytic bacteria isolated from *Moringa oleifera* leaves

S. No.	Isolate No.	Cellulase activity	Amylase activity	Esterase activity
1.	JM-1a	Negative	Negative	Negative
2.	JM-1b	Negative	Negative	Negative
3.	JM-1c	Negative	Negative	Negative
4.	JM-1d	Negative	Negative	Negative
5.	JM-1e	Negative	Negative	Negative
6.	JM-2a	Negative	Negative	Negative
7.	JM-2b	Negative	Negative	Negative
8.	JM-2c	Negative	Negative	Negative
9.	JM-2d	Negative	Negative	Negative
10.	JM-2e	Negative	Negative	Negative
11.	NM-1a	Negative	Negative	Negative
12.	NM-1b	Negative	Negative	Negative
13.	NM-1c	Negative	Negative	Negative
14.	NM-1d	Negative	Negative	Negative
15.	NM-1e	Negative	Negative	Negative
16.	NM-2a	Negative	Negative	Negative
17.	NM-2b	Negative	Negative	Negative
18.	NM-2c	Negative	Negative	Negative
19.	NM-2d	Negative	Negative	Negative
20.	NM-2e	Negative	Negative	Negative

Table 05:
Biochemical tests of endophytic bacteria isolated from *Moringa oleifera* leaves

S. No.	Isolate No.	Catalase test	Coagulase test	Oxidase test	V P Test	ONPG Test	Urease test	Arginine utilization test	Sugar fermentation test		
									Sucrose	Maltose	Lactose
1.	JM-1a	Positive	Negative	Positive	Negative	Positive	Negative	Negative	Negative	Negative	Negative
2.	JM-1b	Positive	Negative	Positive	Negative	Positive	Negative	Negative	Negative	Negative	Negative
3.	JM-1c	Positive	Negative	Positive	Negative	Positive	Negative	Negative	Negative	Negative	Negative
4.	JM-1d	Positive	Negative	Positive	Negative	Positive	Negative	Negative	Negative	Negative	Negative
5.	JM-1e	Positive	Negative	Positive	Negative	Positive	Negative	Negative	Negative	Negative	Negative
6.	JM-2a	Positive	Negative	Positive	Negative	Positive	Negative	Negative	Negative	Negative	Negative
7.	JM-2b	Positive	Negative	Positive	Negative	Positive	Negative	Negative	Negative	Negative	Negative
8.	JM-2c	Positive	Negative	Positive	Negative	Positive	Negative	Negative	Negative	Negative	Negative
9.	JM-2d	Positive	Negative	Positive	Negative	Positive	Negative	Negative	Negative	Negative	Negative
10.	JM-2e	Positive	Negative	Positive	Negative	Positive	Negative	Negative	Negative	Negative	Negative
11.	NM-1a	Positive	Negative	Positive	Negative	Positive	Negative	Negative	Negative	Negative	Negative
12.	NM-1b	Positive	Negative	Positive	Negative	Positive	Negative	Negative	Negative	Negative	Negative
13.	NM-1c	Positive	Negative	Positive	Negative	Positive	Negative	Negative	Negative	Negative	Negative
14.	NM-1d	Positive	Negative	Positive	Negative	Positive	Negative	Negative	Negative	Negative	Negative
15.	NM-1e	Positive	Negative	Positive	Negative	Positive	Negative	Negative	Negative	Negative	Negative
16.	NM-2a	Positive	Negative	Positive	Negative	Positive	Negative	Negative	Negative	Negative	Negative
17.	NM-2b	Positive	Negative	Positive	Negative	Positive	Negative	Negative	Negative	Negative	Negative
18.	NM-2c	Positive	Negative	Positive	Negative	Positive	Negative	Negative	Negative	Negative	Negative
19.	NM-2d	Positive	Negative	Positive	Negative	Positive	Negative	Negative	Negative	Negative	Negative
20.	NM-2e	Positive	Negative	Positive	Negative	Positive	Negative	Negative	Negative	Negative	Negative

endophytic bacteria against different Gram positive bacteria have been showed Isolated endophytic bacteria from *Moringa oleifera* showed antibacterial activity as 75 per cent isolates inhibited growth of *Staphylococcus aureus* and no isolate had inhibited growth of *Streptococcus pyogenes* and *Bacillus cereus* (Table 7).

The *in vitro* antibacterial activity of endophytic bacteria against different Gram negative bacteria have been showed Isolated endophytic bacteria from *Moringa oleifera* showed antibacterial activity as 85 per cent of isolates inhibited the growth of *Salmonella* Typhimurium and none of the isolates had inhibited growth of *Klebsiella pneumoniae* and *Escherichia coli* (Table 8).

Total 20 isolates from *Moringa oleifera* were tested for antibacterial activity. Over all screening report is presented in Table 9. Out of 20 isolates from *Moringa oleifera*, 15 isolates were effective against *Staphylococcus aureus*. Not a single isolate from *Moringa oleifera* had shown activity against *Streptococcus pyogenes* and *Bacillus cereus*. 17 isolates were effective against *Salmonella* Typhimurium. Not a single isolate from *Moringa oleifera* were effective against *Klebsiella pneumoniae* and *Escherichia coli.*

The overall *in vitro* antibacterial sensitivity test

Table 7:
In vitro antibacterial activity of endophytic bacteria isolated from *Moringa oleifera* leaves against Gram positive bacteria

S. No.	Isolate No.	*Staphylococcus aureus*	*Streptococcus pyogenes*	*Bacillus cereus*
1.	JM-1a	S	R	R
2.	JM-1b	S	R	R
3.	JM-1c	S	R	R
4.	JM-1d	R	R	R
5.	JM-1e	R	R	R
6.	JM-2a	S	R	R
7.	JM-2b	S	R	R
8.	JM-2c	S	R	R
9.	JM-2d	S	R	R
10.	JM-2e	S	R	R
11.	NM-1a	S	R	R
12.	NM-1b	S	R	R
13.	NM-1c	S	R	R
14.	NM-1d	S	R	R
15.	NM-1e	S	R	R
16.	NM-2a	S	R	R
17.	NM-2b	R	R	R
18.	NM-2c	S	R	R
19.	NM-2d	R	R	R
20.	NM-2e	R	R	R

Table 8 :
In vitro antibacterial activity of endophytic bacteria isolated from *Moringa oleifera* leaves against Gram negative bacteria

S. No.	Isolate No.	*Salmonella* Typhimurium	*Klebsiella pneumoniae*	*Escherichia coli*
1.	JM-1a	S	R	R
2.	JM-1b	S	R	R
3.	JM-1c	S	R	R
4.	JM-1d	S	R	R
5.	JM-1e	S	R	R
6.	JM-2a	S	R	R
7.	JM-2b	S	R	R
8.	JM-2c	S	R	R
9.	JM-2d	S	R	R
10.	JM-2e	S	R	R
11.	NM-1a	R	R	R
12.	NM-1b	S	R	R
13.	NM-1c	S	R	R
14.	NM-1d	S	R	R
15.	NM-1e	S	R	R
16.	NM-2a	R	R	R
17.	NM-2b	S	R	R
18.	NM-2c	S	R	R
19.	NM-2d	S	R	R
20.	NM-2e	R	R	R

results showed that sensitivity was observed by the endophytic bacteria isolated from *Moringa oleifera*. Most of the isolates from *Moringa oleifera* had shown antibacterial activity against Gram positive (*Staphylococcus aureus*) and gram negative bacteria (*Salmonella* Typhimurium). This suggests that metabolites of endophytic bacteria might have diffused in the culture medium and suppressed the growth of pathogenic bacteria. The bacterial strains secrete different types of natural products to inhibit or kill a wide variety of harmful disease causing agents including, bacteria, fungi, viruses and protozoans that affect humans and animals (Kumar *et al*., 2016). The bioactive compound could easily move into the bacterial cell membrane *via* the general bacterial porins, which might be responsible for several metabolic functions of the cell or they may enter from various pores in the outer cell membrane of bacteria, resulting in the leakage of internal substances to the outside, causing lysis of cell and death (Islam *et al*., 2018).

Table 9 :
Over all *in vitro* antibacterial activity of endophytic bacteria isolated from *Moringa oleifera* leaves

S. No.	Bacteria	No. of isolate which showed sensitivity
1.	*Staphylococcus aureus*	15
2.	*Streptococcus pyogenes*	0
3.	*Bacillus cereus*	0
4.	*Salmonella* Typhimurium	17
5.	*Klebsiella pneumoniae*	0
6.	*Escherichia coli*	0

It is concluded from this investigation that phenetic characterization and biochemical tests indicated the presence of endophytic bacteria in the leaves of *Moringa oleifera*. Gram negative rods were present in the leaves of *Moringa oleifera*. The endophytic bacteria obtained from *Moringa oleifera* had shown antibacterial activity against *Staphylococcus aureus* (Fig. 7) and *Salmonella* Typhimurium (Fig. 8).

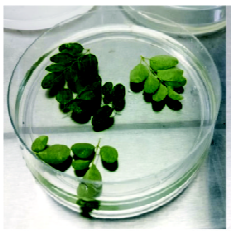
Fig. 1


Fig. 2


Fig. 3

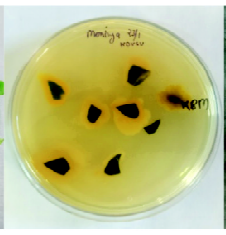
Fig. 4


Fig. 5


Fig. 6

Fig 1: Washing of *Moringa oleifera* leaves. **Fig 2:** Drying of *Moringa oleifera* leaves. **Fig 3:** Cut pieces of *Moringa oleifera* leaves. **Fig 4:** Growth of endophytic bacteria from *Moringa oleifera* leaves on King's B media . **Fig 5:** Growth of endophytic bacteria from *Moringa oleifera* leaves on sheep blood agar. **Fig 6:** Growth of endophytic bacteria from *Moringa oleifera* leaves in BHI broth.

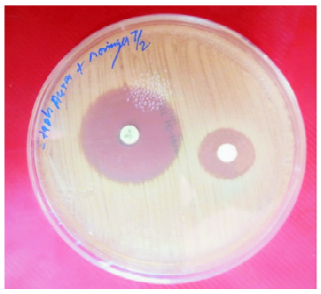

Fig. 7

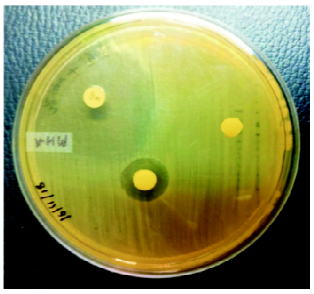

Fig. 8

Fig 7:
In vitro antibacterial activity of endophytic bacteria isolated from *Moringa oleifera* leaves against *Staphylococcus aureus,* **Fig 8 :***In vitro* antibacterial activity of endophytic bacteria isolated from *Moringa oleifera* leaves against *Salmonella* Typhimurium.

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Research Article

IN VITRO ANTIOXIDANT POTENTIAL OF *RHODODENDRON ARBOREUM* LEAF EXTRACTS

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ABSTRACT

The study was carried out to evaluate the in vitro antioxidant potential of hydroethanolic and hydromethanolic extract of *Rhododendron arboreum*. Hydroethanolic extract showed 90.55% and hydromethanolic extract 84.14% DPPH scavenging activity at 250μg/ml concentrations, respectively. The IC_{50} values of hydroethanolic and hydromethanolic extracts were 65.47μg/ml and 65.40 μg/ml, respectively. ABTS scavenging activity of *R. arboreum* showed a significant (P< 0.05) increase in antioxidant activity viz., 83.61 % whereas hydromethanolic extract showed 78.41% at 250 μg/ml. The IC_{50} values of hydroethanolic and hydromethanolic extracts were 70.81 μg/ml, 70.94μg/ml, respectively. On assay of nitric oxide synthetase activity, the maximum activity was observed in hydroethanolic extract followed by hydromethanolic extracts of *Rhododendron arboreum* viz., 81.22% and 77.28%, respectively, at 500 μg/ml. The IC_{50} values for hydroethanolic and hydromethanolic extracts of *Rhododendron arboreum* were 144.13 μg/ml and 154.34 μg/ml, respectively, which showed potent antioxidant potential. Thus, both the extract showed potent antioxidant potential, however, hydroethanolic extract showed more potent antioxidant activity than hydromethanolic extract.

Key words: Antioxidant potential, DPPH assay, *Rhododendron arboretum,* ABTS assay, nitric oxide synthetase

INTRODUCTION

Rhododendron arboretum, commonly kmnown as *Burans,* is found in North Central India, Himalayas, Areas of Kashmir, Assam and Manipur and specifically can be grown only in high altitude regions ranging from 600 m above sea level to above and grow with moderate shade and is better grown in conservation (Srivastava, 2012; Gill *et al.,*2015).

Green leaves contain glycoside, ericolin (arbutin) ($C_{12}H_{16}O_7$), ursolic acid ($C_{30}H_{48}O_4$), alpha-amyrin ($C_{30}H_{50}O$), epifriedelinol ($C_{30}H_{52}O$) and new triterpenoids named campanulin, quercetin and hyperoside ($C_{21}H_{20}O_{12}$). The leaves also contain the flavone glycoside and dimethyl ester of terephthalic and contain flavonoids (Rawat *et al.,* 2018).

Rhododendron arboreum has certain scientifically validated properties like anti-inflammatory and anti-nociceptive activity (Nisar *et al.,* 2016) andhepatoprotective activity(Verma *et al.,* 2011). Less literature is available on *Rhododendron arboreum in-vitro* antioxidant property in leaves and hence this study is undertaken to evaluate its antioxidant potential by determining in vitro test.

The study was undertaken for evaluation of DPPH radical scavenging activity, ABTS scavenging activity and nitric oxide synthetase activity in *Rhododendron arboreum*hydroethanolic (60:40) and hydromethanolic (50:50) leaf extract.

MATERIALS AND METHODS

DPPH radical scavenging assay

The scavenging activity of various extracts on 2,2-Diphenyl-2 picrylhydrazyl (DPPH) free radicals was evaluated by the method as described by Williams *et al.* (1995). An increased level of free radical scavenging activity is an indicator of increased anti-oxidant property. The absorbance of the samples was recorded at 517 nm against reaction blank. The radical scavenging activity was assessed by the following formula.

Radical scavenging activity (%) = $[1-(A_1/A_o)]\times 100$;

Where, A_0 and A_1 – absorbance of control and test, respectively.

ABTS radical scavenging assay

2, 2'- azinobis (3- ethylibenzthiazoline)- 6-sulfonic acid (ABTS) assay was used to determine the level of antioxidant status of the extract. The principle of the assay is that it relies on the antioxidant capability of the samples to inhibit the oxidation of ABTS to $ABTS^+$ radical cation. ABTS radical scavenging assay was performed following the method of Re *et al.,(* 1999). The % ABTS inhibition was calculated by the following formula:

% ABTS inhibition = $[1-(A_1/A_O)] \times 100$

Where, A_o& A_1- Absorbance of control and test respectively.

Nitric oxide radical scavenging assay

The assay is performed by using Griess reaction as described by Mudal and Goli (2005). 500 µl samples of different concentrations from 50-500 µg/ml were prepared. Reaction control was prepared by adding water instead of extract. 500 µl of sodium nitroprusside solution was added to the solvent and kept at 25°C for 2.5 hrs. After 2.5 hrs, the reaction mixture was mixed with 1 ml of griess reagent viz (100 ml contains 1 gm of 1%

sulphanilamide and 2 ml o-phosphoric acid and naphthyl ethylene dihydrochloride 0.1%, 100mg). The reaction was spectrophotometrically measured at 546 nm and the % inhibition of nitric oxide generated was measured by comparing the absorbance values of control and test. Ascorbic acid was used as a standard reference compound. Percent nitric oxide scavenging activity was calculated as:

% Nitric oxide scavenging activity = $[1-(A_1/A_O)] \times 100$

Where, A_o & A_1 - Absorbance of control and test respectively.

RESULTS AND DISCUSSIONS

DPPH radical scavenging activity of RAHE in comparison to the standard reference compound ascorbic acid is mentioned in the Table 1. The study revealed that hydroethanolic extract showed 90.55% and hydromethanolic extract 84.14% DPPH scavenging activity at 250μg/ml concentrations, respectively, which indicated marked antioxidant potential of the extract. IC_{50} values of hydroethanolic and hydromethanolic extracts were 65.47ug/ml and 65.40 ug/ml respectively. The findings of the study are correlated with the findings of Painuli *et al.* (2018) who also recorded DPPH activity of 91.67% in ethanolic and 88.56% activity in methonolic extracts of *R. arboreum* leaves. The antioxidant activity of the plant might have been due to the presence of various flavonoids and phenols in the extract as reported in this study. Hydroethanolic extract of the compound showed significantly (P<0.05) higher antioxidant activity than hydromethanolic extract indicating a greater potential in this study.

The DPPH is a potent radical and a scavenger for various free radicals andhence the rate of reduction ofany chemical reaction upon addition of DPPH is considered as an indicator of the radical scavenging property of the reactant. DPPH radicals show deep violet color and when neutralized at 517 nm due to strong absorption, it turns back to pale yellow. The property of change in color of the radical allows to monitor the reaction and spectrophotometerically measure the reaction at 517 nm (Geng *et al.,* 2015).

The values of ABTS scavenging activity in hydroalcoholic extracts of *R.arboreum* are mentioned in the Table 2 with reference to the standard compound viz. Trolox. ABTS is commonly used as a substrate with H2O2 for peroxidase enzyme or even alone with multicopper oxidase enzyme. It is used as it allows the reaction kinetics of peroxidases themselves to be followed, thus, it can be indirectly used to track the reaction kinetics of any H_2O_2 producing enzyme or to evaluate the amount of H_2O_2 available in the sample. The compound is mainly preferred as itsupports the reaction with $H_2O_2$and turning green colored ABTS to colorless by the end of the experiment. The hydroethanolic extract of *R. arboretums,* however, a significant (P< 0.05) increase in antioxidant activity viz., 83.61% whereas hydromethanolic extract showed less antioxidant activity than the former extract viz., 78.41% at 250 μg/ml. The IC_{50} values of hydroethanolic, hydromethanolic extract and trolox were 70.81 μg/ml, 70.94μg/ml and 10.7μg/ml, respectively. The findings of the study are indicative of antioxidant potential of the extract and can be correlated with the findings of Painuli *et al*. (2018) who reported 96.55% activity in methanolic and 97.12% in ethanolic extracts of *R. arboretum* leaves.

The values of nitric oxide synthetase activity of hydroethanolic and hydromethanolic extracts of *R. arboreum* are depicted in the Table 3. The maximum

Table 1:
Effect of hydroalcoholic extracts of *R. arboreum* on DPPH radical scavenging activity.

	Percent inhibition		
Concentration (μg/ml)	Hydroethanolic	Hydromethanolic	Ascorbic acid
250	90.55±0.11a	84.14±0.08a	97.33±0.34a
125	58.01±0.14b	53.30±0.12b	91.43±0.20b
62.5	31.86±0.24c	26.08±0.09c	87.16±0.28c
31.25	14.63±0.22d	17.56±0.13d	43.27±0.01d
15.62	9.65±0.19e	8.08±0.10e	24.43±0.09e
7.81	5.49±0.07f	4.51±0.06f	8.22±0.00f
3.91	2.82±0.02g	3.08±0.03g	5.61±0.00g
IC50 (ug/ml)	65.47	65.40	37.83

Table 2:
Effect of hydroalcoholic extracts of *R. arboreum* on ABTS radical scavenging activity.

	Percent inhibition		
Concentration (μg/ml)	Hydroethanolic	Hydromethanolic	Trolox
250	83.61±0.16a	78.41±0.46a	97.18±0.25a
125	54.59±0.28b	45.52±0.28b	70.32±0.36b
62.5	23.59±0.19c	24.68±0.24c	47.19±0.28c
31.25	11.66±0.05d	17.24±0.11d	35.73±0.21d
15.62	5.53±0.22e	4.46±0.18e	28.10±0.20e
7.81	4.34±0.17f	3.49±0.18e	19.91±0.06f
3.91	2.73±0.16g	2.16±0.05f	11.12±0.052g
IC_{50} (μg/ml)	70.81	70.94	10.7

Table 3:
Effect of hydroalcoholic extracts of *R. arboreum* on nitric oxide synthetase activity.

	Percent inhibition (%)		
Concentration (μg/ml)	Hydroethanolic	Hydromethanolic	Ascorbic acid
500	81.22±0.10a	77.28±0.15a	99.51±0.15a
250	57.36±0.38b	52.65±0.13b	97.33±0.34b
125	32.56±0.02c	27.14±0.25c	91.43±0.20c
62.5	15.09±0.05d	11.78±0.06d	87.16±0.28d
50	9.21±0.06e	6.36±0.15e	60.30±0.42e
IC_{50} (μg/ml)	144.13	154.34	37.83

In Table 1, 2 and 3, mean values bearing different superscripts i.e., a,b,c,d,e and f differ significantly (P<0.03) when compared vertically in a column.

activity was observed in hydroethanolic extract followed by hydromethanolic extracts of *R. arboretum* viz., 81.22%, 77.28%, respectively, at 500 μg/ml. Ascorbic acid was taken as the standard reference drug which showed 99.51% activity at 500 μg/ml. The IC_{50} values for hydroethanolic and hydromethanolic extracts of *R. arboretum* were 144.13 μg/mland 154.34 μg/ml,respectively, which indicated a significant ($P<0.05$) antioxidant activity of the extracts. Similar findings were observed in studies conducted by Acharya *et al.* (2011)who demonstrated significantly higher activity of nitric oxide synthetase in ethanolic extracts as compared to cold and hot water extracts of *R. arboreum* flowers with EC_{50} value of 41.7 μg/ml.

Nitric oxide at cellular level acts as a signaling molecule and is synthetized from the precursor L-arginine in a reaction catalyzed by the nitric oxide synthetase enzyme. Any pathophysiological condition in the body leading to low production of nitric oxide in the body is indicative of various detrimental health conditions like hypertension, Alzheimer disease, atherosclerosis, diabetes mellitus, hypoxia, fibrosis, cancer, myocardial infarction, stroke etc. (Dawoud and Malinski, 2018). The improved antioxidant activity observed in the extracts of *R. arboreum* is indicative of its potent antioxidant activity due to presence of various flavonoids and phenols in the extract as also observed in this study. It is concluded from this study that both the extract showed potent antioxidant potential and hydroethanolic extract showed more potent antioxidant activity than hydromethanolic extract.

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Research Article

CANDESARTAN AMELIORATED ARSENIC INDUCED TESTICULAR TOXICITY IN RATS

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ABSTRACT

The study was conducted to evaluate the ameliorative effect of candesartan, an angiotensin II receptor blocker in arsenic-induced testicular toxicity in rats. Total study period was 90 days. Adult male Wistar rats were divided into 4 groups (n=8). Group I was kept as control and was given only potable drinking water. Group II was given 1 mg/kg candesartan cilexetil in Carboxymethylcellulose (CMC) last 30 days. Group III, arsenic treated group was given 50 ppm sodium arsenite in drinking water for 90 consecutive days. Group IV was administered with 50 ppm arsenic for 90 days along with candesartan from 61-90 days. All untreated group were given vehicle CMC for last thirty days. Different sperm quality parameters like total sperm count, sperm liveability, sperm abnormality percentage and histopathological alterations of testes were studied. Histological alterations and reduction in sperm count, liveability and increase in abnormality were observed in arsenic treated animals. Candesartan treatment could restore the testicular damage and sperm quality deterioration in the arsenic treated rats.

Key words: Arsenic, candesartan, testicular toxicity, rats.

INTRODUCTION

Millions of people in several countries live at a risk of arsenic exposure through contaminated drinking water exceeding the WHO recommended level of 10 ppb (Ellinsworth, 2015). Arsenic affects various organ systems through multiple mechanisms. The deteriorative effect of arsenic on the male reproductive system and the semen quality were previously described. Acute or chronic exposure of arsenic produces damage to the male reproductive system by reducing the testicular weight, impairing steroidogenesis and spermatogenesis, increasing inflammation and oxidative stress and causing male reproductive dysfunction (Renu *et al.,* 2018). Arsenic exposure can activate various signalling pathways like ERK/AKT/NF-kB (Huang *et al.,* 2016), bind directly to sulphydryl group of sperm nuclear chromatin that mediates reproductive damage (Kim & Kim, 2015), and disrupts spermatogenesis by inducing apoptosis (Trevino *et al.,* 2018).

Candesartan, a highly potent and selective antagonist of the angiotensin II receptor subtype 1, administered orally as candesartan cilexetil which on hydrolysis into candesartan active moiety produces antihypertensive effect in a dose-dependent manner (Sever, 1997). Candesartan was also studied for its pleotropic effects as an anti inflammatory, anti oxidative, anti atherotic and protective against Alzheimer's disease (Trigiani *et al.,* 2018). ARBs like candesartan and losartan reversed thioacetamide-induced low grade testicular toxicity in rats (Faddladdeen *et al.,* 2019). Candesartan could prevent cisplatin induced testicular damage in rat testes by regulating the expression of nephrin-podocin complex (Enatsu *et al.,* 2015). Angiotensin Converting Enzyme Inhibitors like captopril and telmisartan reversed cadmium-induced testicular damage in rats (Fouad & Jresat, 2013). Taking these into consideration, ameliorative effect of candesartan was evaluated in arsenic-induced testicular toxicity in rats.

MATERIALS AND METHODS

Chemicals

Sodium arsenite was purchased from sigma Aldrich and candesartan cilexetil was purchased from Santa cruz technology, USA. All other chemicals used were of analytical grade from Sigma-Aldrich, St. Louis; SRL Chemicals, India.

Animals

Apparently healthy male Wistar rats were procured from the Laboratory Animals Resource Section of the institute. The animals were kept in the laboratory animal house for 7 days for acclimatization before the start of the experiment and fed standard pellet feed and potable drinking water. Animals were handled and all experiments were conducted as per the recommended guidelines of the Institute Animal Ethics Committee.

Dose selection

In the present study arsenic as sodium metaarsenite at the level of 50 ppm was exposed through drinking water to mimic the natural conditions of arsenic

exposure (Khuman *et al.,* 2016). Candesartan administered orally @ 1 mg/kg bw orally for 30 days produced antihypertensive, vasculoprotectant and antifibrotic effects in rats exposed to arsenic subchronically (Khuman *et al.,* 2016). Accordingly, candesartan @ 1 mg/kg bw was selected and administered daily orally for 30 days.

Experimental design

Male Wistar rats were divided randomly into 4 groups. Animals of Group I and II received only potable drinking water, while animals of Group III and IV received 50 ppm sodium arsenite, through drinking water for 90 consecutive days. Further, the animals of Groups II and IV were administered candesartan (1 mg/kg bw as aqueous suspension in 0.5% carboxy methyl cellulose) by oral gavage once daily during the last 30 days, i.e., from day 61 to day 90 of arsenic exposure. Daily feed and water consumption and weekly body weight were recorded.

Group (n=8)	Treatment	Treatment Schedule	
		1-60 days	61-90 days
I	Control	-	CMC
II	Candesartan	-	Candesartan in CMC
III	Arsenic	Sodium arsenite	Sodium arsenite + CMC
IV	Arsenic + Candesartan	Sodium arsenite	Sodium arsenite + Candesartan in CMC

CMC: Carboxymethylcellulose

Sample collection

All animals from each group were sacrificed by complete bleeding of the abdominal aorta under anesthesia. Testes were collected weighed and stored in modified Davidson's fluid and cauda epididymis was isolated for collecting sperms.

Study on the effect on male reproductive characteristics

Epididymal total sperm count

The epididymal sperms obtained after slaughter were incubated at 35°C, which is the optimum temperature of rat epididymal sperm. The epididymal fluid was then diluted upto 5 ml in pre-warmed (35°C) Dulbecco's PBS. The spermatozoa were counted by hemocytometer using the Improved Neubaur's (Deep 1/ 10mm. LABART, Germany) chamber as described by Pant and Srivastava (2003) using semen diluting fluid (5 gm sodium bicarbonate, 1 ml formalin and 99 ml distilled water).

Total Sperms/ml = (Average number of sperm per chamber) x 10^3 x (Dilution Factor)

Liveability and abnormal sperm

Percent live spermatozoa was estimated by differential staining technique using Eosin-Nigrosin staining (NE). These slides were also used for estimating the percent abnormal sperm morphologically on the basis of observable abnormalities of head, neck, mid-piece and tail region of the spermatozoa. A drop of epididymal semen was taken on a clean, grease free pre warmed glass slide. 4-5 drops of Eosin-Nigrosin stain (1% Eosin and 5% Nigrosin in 3% sodium citrate solution) was placed near the semen drop. Epididymal semen and stain were mixed gently using a blunt fine glass rod. After 30 seconds a thin smear was made on a clean, grease free glass slide. The smear was examined under oil immersion objective. A total of 200 spermatozoa were counted in a slide. The stained and partially stained spermatozoa were considered as dead.

Sperm abnormality

Two hundred spermatozoa (heads only or intact sperm) per rats were evaluated for head and/or flagellar defects by microscopy (100X). Classifications of individual spermatozoa were: a) normal, b) normally shaped head separated from flagellum, c) misshapen head separated from flagellum, d) misshapen head with normal flagellum, e) misshapen head with abnormal flagellum, f) degenerative flagellar defect(s) with a normal head, and g) other flagellar defects(s) with a normal head(Pant and Srivastava, 2003).

Histopathology of testes

The testes fixed in modified Davidson's fluid were dehydrated in ascending alcohol series and embedded in paraffin wax. Approximately 5 µm thick sections were made, deparaffinised by dipping in xylene for 15 min and stained with haematoxylin and eosin for examining the histological alterations under light microscope.

Statistical analysis

Statistical analysis was carried out using GraphPad Prism 5 software (GraphPad Software Inc., San Diego, CA). One way ANOVA followed by Newman-Keuls multiple comparison *post-hoc* test was used. *P-value* <0.05 was considered as statistically significant.

RESULTS

The total sperm count (Fig. 1) was significantly reduced in arsenic treated animals compared to control group (P>0.05). The sperm count on arsenic exposure was significantly increased in As + Candesartan group compared with As-treated group. While comparing control and candesartan group, there was no significant difference shown in the sperm count.

Total sperm abnormalities (Fig. 2) in arsenic treated group showed a significant increase in arsenic-treated animals compared to control. Sperm abnormality was significantly reduced by candesartan treatment in As-exposed rats. The percentage of live sperm (Fig. 3) was significantly reduced in arsenic treated animals compared to control. The live sperm percentage was increased significantly in As+candesartan group which indicates the restoration of sperm liveability by candesartan in arsenic exposed rats. Compared to

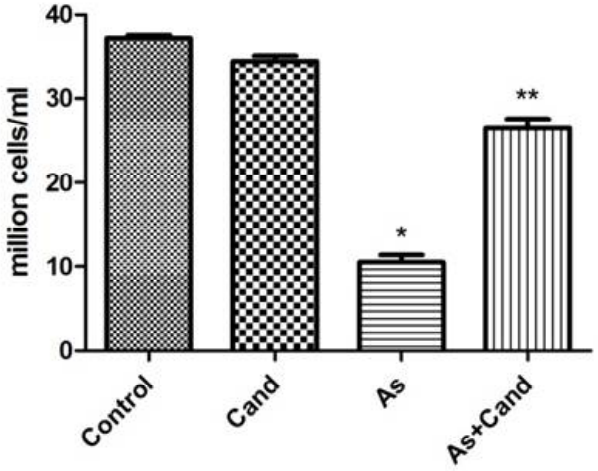

Fig. 1. Total sperm count (million cells/ml)

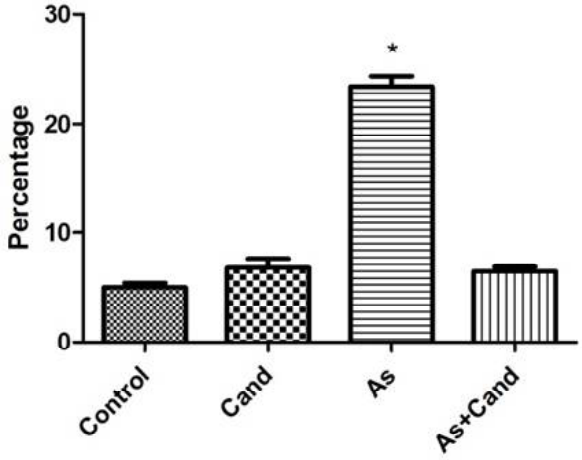

Fig. 2. Sperm abnormality (percentage)

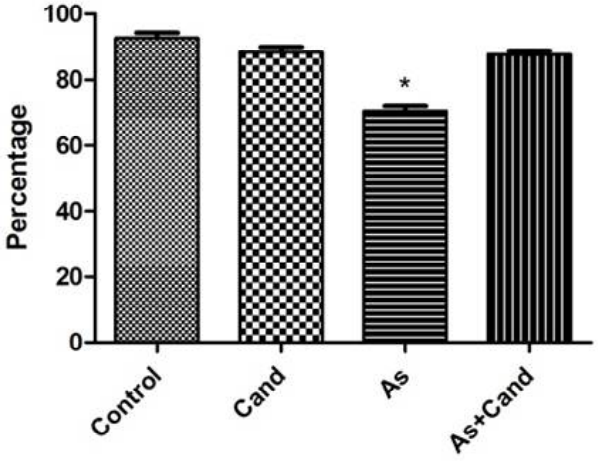

Fig. 3. Live sperms (percentage)

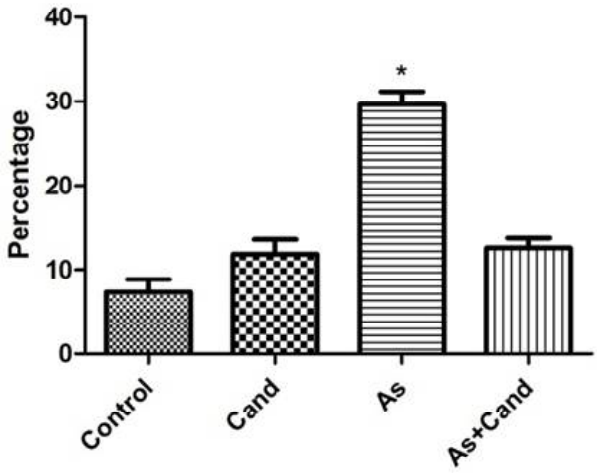

Fig. 4. Dead sperms (percentage)

Fig. 1 to 4. Effect of arsenic on total sperm count (Fig-1), Sperm abnormalities (Fig-2), live and dead sperm percentage(Fig 3&4) Bars bearing different superscripts vary significantly between groups. (Cand: Candesartan, As: Arsenic, As+Cand: Arsenic + Candesartan)

control, there was no significant difference in live sperm percentage in candesartan treated group. Similar trend was observed in dead sperm percentage (Fig. 4).

Histopathological examination of testes sections showed intact features in control (Fig. 5a) and candesartan (Fig. 5b) groups. In arsenic exposed group (Fig. 5c), testicular oedema was visible. This was damage was reversed to normal histological appearance on candesartan treatment in group IV (Fig. 5d).

DISCUSSION

The total sperm count was reduced significantly on subchronic arsenic exposure in our study. Epidemiological studies in arsenic rich areas revealed higher incidence of male infertility problems (Sengupta *et al.,* 2013). A decrease in sperm count and sperm motility, along with an increase in abnormal sperm, was observed in arsenite-exposed mice (Pant *et al.,* 2004). Similiarly, the sperm abnormality and dead sperm percentage was significantly higher in arsenic exposed rats in our study. Deteriorative effects of arsenic on spermatogenesis occur by multiple mechanisms which were extensively studied in experimental animals and by epidemiological investigations (Renu *et al.,* 2018). Arsenite exposure decreased the level of luteinizing hormone (LH), follicle stimulating hormone (FSH) and testosterone with degeneration of stage VII germ cells in mice (Sarkar *et al.,* 2003).

Candesartan showed no significant change in any of the sperm parameters compared to control group. Interestingly, arsenic-induced deterioration in sperm quality parameters could be significantly ameliorated

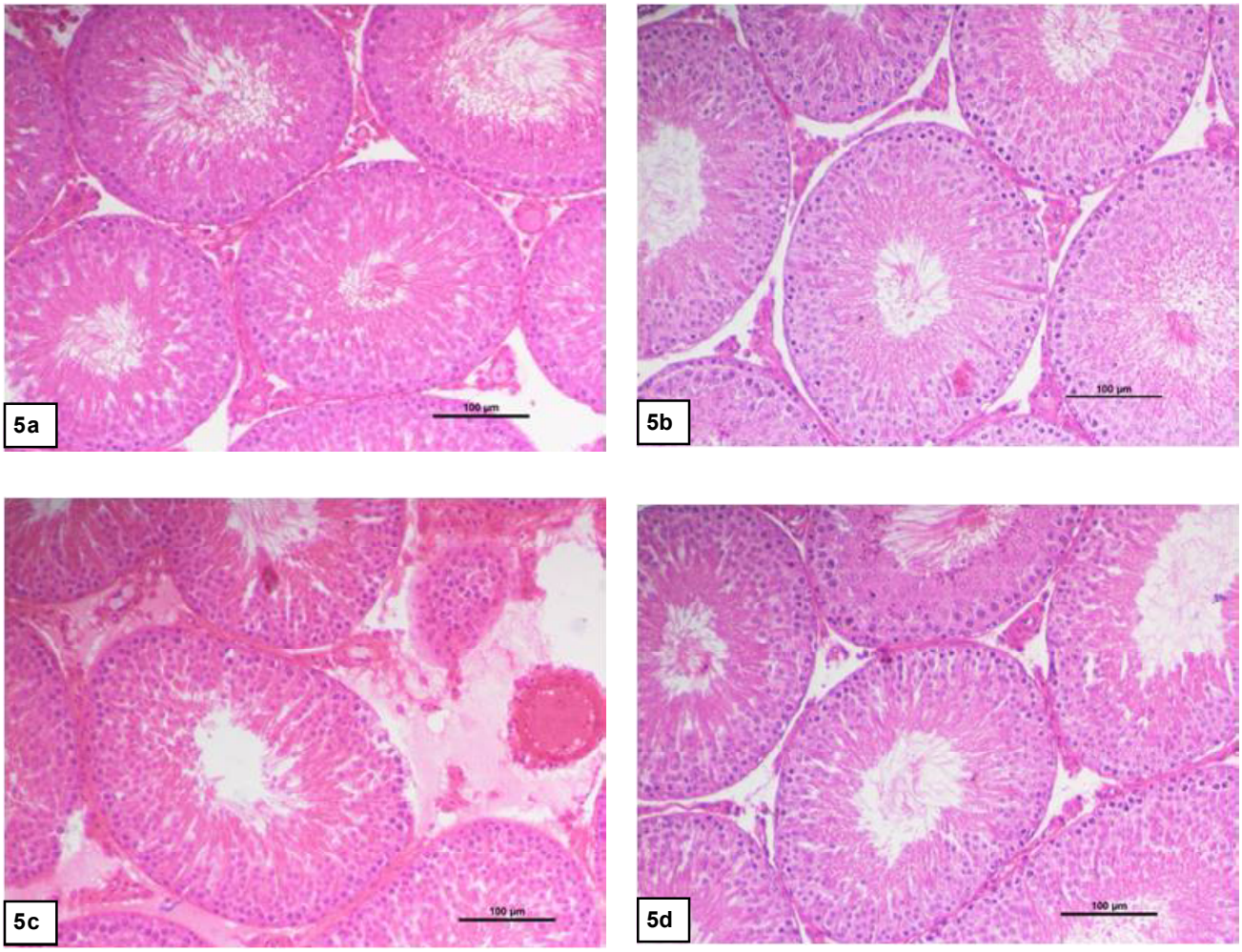

Fig. 5. 5a. Control group, Testis, H&E x 200, Normal testis section showing intact histology. **Fig. 5b. Candesartan group**, Testis, H&E x 200, Normal testis section showing showing intact histology. **Fig. 5c. Arsenic group**, Testis, H&E x 200, testis section showing interstitial oedema. **Fig. 5d. Arsenic + Candesartan group**, Testis, H&E x 200, Normal testis section showing intact histology.

by candesartan. Candesartan treatment in arsenic exposed animals increased the sperm count, reduced sperm abnormalities and increased live sperm percentage at a level comparable to control. Candesartan reversed cisplatin induced-gonadal damage via antioxidant, anti-inflammatory, anti-apoptotic actions (Sherif and Sarhan, 2019) and regulating the expression of nephrin-podocin complex in testes (Enatsu *et al.,* 2015).

Histopathological analysis of testes sections revealed intact testicular architecture in candesartan treated group. In arsenic exposed animals, there was testicular oedema, which was restored to normal intact histology with candesartan treatment. Arsenic toxicity resulted in atrophic damage in testis due to degenerative changes in spermatogenic and leydig cells (Ahmad *et al.,* 2008). Arsenic induced testicular damage characterised by widespread necrosis, vacuolization of seminiferous tubular cells, marked reduction in spermatogenesis and interstitial tissue oedema were ameliorated by telmisartan treatment (Fouad *et al.,* 2015). Due to its capability to ameliorate arsenic-induced male reproductive toxicity, candesartan can be explored as a potential therapeutic agent for male infertility cases in arsenic rich areas. Further studies are required to evaluate the possible molecular mechanisms by which candesartan could reverse the testicular damage and its effectiveness in human and animals.

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Short Communication

PHYTOCHEMICAL ANALYSIS OF *RHODODENDRON ARBOREUM.* LEAF EXTRACTS

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ABSTRACT

This study was undertaken for qualitative phytochemical analysis of hydroethanolic, hydromethanolic, acetone and aqueous extracts of *Rhododendron arboreum* leaves. Plant leaves were collected from the hilly area of Uttarakhand and various extracts were prepared. On phytochemical analysis, different extracts showed the presence of phytochemical groups such as flavonoids, anthraquinones, tannins, sterols, reducing sugars, resins, and proteins. Hydroethanolic extract was found to have the wide range of phytochemical groups in comparison to hydromethanolic, acetone and aques extracts. Aqueous extract contained the least number of phytochemical groups.

Key words: Leaf extracts, phytochemical analysis, *Rhododendron arbreum*

High altitude plants are very well used all over the world for treatment and prevention of diseases. These high altitude regions or ecosystem are considered to be the areas as hotspots of medicinal plant diversity but still are neglected in respect to research and development. Major hindrance is the harsh climatic conditions causing inaccessibility to these plants. A survey stated that only 20% of the high altitude medicinal plants are used in Indian drug market. Phytochemicals are naturally active chemical compounds present in plant which provide various characteristic antibacterial, antifungal, antioxidant, antidiabetic, antidiarrhoeal and many more desirable action due to secondary metabolites such as phenols, saponins, tannins, coumarins, alkaloids etc. phenols and flavonoids impart anti-oxidant, anti-inflammatory and anti-carcinogenic property etc (Gill *et al*., 2015), tannins have reducing power which leads to prevention of liver injury by inhibition of lipid peroxide, flavonoids also exhibit antioxidant, antiviral, antimicrobial and antiplatelet activities.(Hayyan *et al*., 2016). As negligible reports are available on phytochemical analysis of leaves of *R.arbrium* grown in the hilly terrain of Uttarakhand, this study was undertaken for phytochemical analysis of various leaf extracts of the plant.

Rhododendron arboreum (Burans) leaves (about 4 kg) were collected from D.S.B. Campus Nainital, located at 1370 meters above the sea level, and nearby places in vicinity of 5-6 kms during the November month (Temp: 20^0 C) from about 30 trees Identification and authentication of the plant was confirmed by Department of Biological Sciences of G.B. Pant University of Agriculture and Technology, Pantnagar.

Leaves of *Rhododendron arboreum* were separated and shade dried in laboratory for 22 days followed by further drying in a fan incubator at 37^0C for 6-7 days to remove the extra moisture present in the leaves. The dried leaves were further grinded in a mixer to obtain a fine homogenous powder of light brown color. The powder was further stored in seeded and covered plastic bags and kept in dry place for further use in the required experiments. Dried powder of the extract was soaked in the prepared solvents and allowed to stand for 24 hours. The filtrate obtained on the next day was filtered using muslin cloth and filtered again with the help of Whatman filter paper no. 42. The obtained filtrate was subjected to rotatory vacuum evaporator at 40^o-50^o C and further dried in incubator at 37^oC-39^oC for 48 hrs. From the obtained dry powder percentage yield of the extract was calculated which further used for *in vivo* and *in vitro* testing in the trial period.

Solvents of hydrethanolic extract was prepared by mixing ethanol and distilled water and ethanol in ratio of 60:40 and hydromethanolic extract was obtained by adding water and methanol in 50:50 ratio. Aqueous and acetone extract were prepared by mixing powder (1gm) in 10 ml of water and 10ml of acetone, respectively. Solution of extracts were prepared by mixing dry extract (1gm) dissolving in 10 ml of solvents and subjected for phytochemical analysis.

The various extracts of *Rhododendren arboreum* dried leaves were subjected to chemical analysis by qualitative methods for presence of important phytochemical groups such as alkaloids, anthraquinones, saponins, flavonoids, tannins, sterol, reducing sugars, glycosides, resins, protein andtriterpenes by of methods (Saklani *et al*., 2011 and Rawat *et al.,* 2018).

Qualitative phytochemical analysis of *R.arboreum* leaves for hydroethanolic, hydromethanolic,

Table 4.1:
Qualitative phytochemical analysis of extract of *Rhododendron arboreum* leaves.

S.No	Phytochemical constituents	Hydoalcoholic		Aqueous	Acetone
		Hydroethanolic	Hydromethanolic		
1.	Alkaloids	-	-	-	-
2.	Anthraquinones	+	+	-	+
3.	Flavonoids	+	+	-	-
4.	Saponins	-	-	-	+
5.	Tannins	+	+	+	-
6.	Sterols	+	-	-	-
7.	Reducing sugars	+	+	+	-
8.	Glycosides	-	-	-	-
9.	Resins	+	-	-	-
10.	Terpenes	-	+	-	-
11.	Proteins	+	-	-	-

acetone and aqueous extracts demonstrated the presence of phytochemical groups such as flavonoids, anthraquinones, tannins, sterols, reducing sugars, resins, and proteins as shown in Table 4.1.

In other reports , green leaves contain glycoside, ericolin (arbutin) ($C_{12}H_{16}O_7$), ursolic acid ($C_{30}H_{48}O_4$), alpha-amyrin ($C_{30}H_{50}O$), epifriedelinol ($C_{30}H_{52}O$) and new triterpenoids named campanulin, quercetin and hyperoside ($C_{21}H_{20}O_{12}$). The leaves also contain the flavone glycoside and dimethyl ester of terephthalic and contain flavonoids (Rawat *et al.,* 2018). Flowers contain quercetin-3-rhamnoside a crystalline compound. HPTLC analysis of flowers showed the presence of 3 biologically active phenols viz. queretin ($C_{15}H_{10}O_7$), rutin ($C_{27}H_{30}O_{16}$) and coumaric acid ($C_9H_8O_3$) (Kashyap and Zaanand, 2016).

The phytochemical analysis of *Rhododendron arboreum* leaves by gas chromatography, mass spectrometry (GC-MS) analysis indicated the presence of important groups of phytoconstituents viz., 22-stigmastem-3-one (14.59%) followed by 1,1,6-trimethyl-3-methylene-2-(3,6,10,13,14-pentamethyl-3-ethanyl-pentadec-4-enye), cyclohexane (12.26%), beta-amyrin (7.62%), and linoleyl alcohol (6.50%) (Sonar *et al.*, 2012).

Upon, qualitative phytochemical analysis of various extracts of *R. arboreum* leaves a greater activity was observed in hydroethanolic extracts in comparison to hydroethanolic followed by acetone and aqueous. Hence, it can be interpreted that the presence of these phytochemical constituents is an indicator of increased activity in hydroethanolic extracts of *R. arboreum* leaves.

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