NUTRITIONAL, PHYTOCHEMICAL AND PHARMACOLOGICAL INVESTIGATION OF BOERHAVIA PROCUMBENS

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NUTRITIONAL, PHYTOCHEMICAL AND PHARMACOLOGICAL INVESTIGATION OF *BOERHAVIA PROCUMBENS*

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ABSTRACT

*Boerhavia procumbens* is an herbaceous, perennial creeping weed and native of Pakistan, belongs to the family Nyctaginaceae. In the folk medicine *B. procumbens* has been used in the treatment of different diseases containing jaundice, hepatitis, and asthma. This research work was design to investigate the crude methanolic extract, its various solvents soluble fractions and isolated compounds of *B. procumbens* for biological activities (*in-vitro* and *in-vivo*) along with chemical composition to provide a valid scientific rationale to its ethno-medicinal uses. In the proximate analysis, the protein (37.46 ± 0.02 %) and inorganic matter (21.25 ± 0.032 %) of the roots were significantly higher (*p* < 0.05) than the aerial parts (2.18 ± 0.04 %, 6.78 ± 0.05). The highest value of Nitrogen-free extract (75.56 ± 0.046 %) was found in aerial parts as compared to roots (31.22 ± 0.05 %). Calcium and potassium were significantly highest (*p*<0.05) elements in the inorganic matter analysis of aerial parts than roots. The essential amino acid, arginine (8.89 ± 0.08 g/100g) and tryptophan (3.49 ± 0.08 g/100g) was found significantly higher (*p* < 0.05) in roots than aerial parts while non-essential amino acid, aspartic acid (31.25 ± 0.08 g/100g) and glutamic acid (25.27 ± 0.06 g/100g) were reported higher in aerial parts than roots of *B. procumbens*. Qualitative phytochemical screening of *B. Procumbens* showed that phenols, flavonoids and cardiac glycoside were present in crude methanolic extract while only phenolic and flavonoidal contents were determined quantitatively in extract and its different solvent fractions. The EtOAc solvent fraction showed significant DPPH radical scavenging activity (IC$_{50}$: 40.24 µg/mL) when compared to standard (Ascorbic acid, IC$_{50}$: 28.78µg/ml). The antibacterial activity of dichloremethane (DCM) solvent fraction (12 ± 0.12 mm) showed good zone of inhibition against *Xanthomonas campestris* while ethyl acetate fraction exhibited maximum activity against *E.coli* (11.8 ± 0.14 mm). In the antifungal activity only n-hexane fraction (23.2 ± 0.20 mm) showed comparable inhibition against *Fusarium oxysporum* to that of standard (23.6 ± 0.16 mm). The DCM fraction (90 %) exhibited a highest cytotoxicity activity against brine shrimp at high concentration (1000 µg/mL) whereas, moderate cytotoxic effect (60 and 10 %) was observed at lower concentrations (100 and 10 µg/mL), respectively. The larvicidal effect against 3rd instar larvae of *Culex quinquefasciatus* of leaf extract and n-hexane fraction showed highest percent mortality (100 % and 96 %) at high concentration (400 µg /ml) with LD$_{50}$ value of 26.84 and 46.98 µg /ml. The DCM fraction showed outstanding phytotoxic potential (50, 63, and 80 %) while n-hexane fraction showed mild activity (13, 20 and 26 %) at the test concentrations of 10, 100 and 1000 µg/mL respectively. In this study six compounds {Indole-3-carboxaldehyde (1), 2-Hydroxybenoic acid (Salicylic acid) (2), 4-Hydroxybenzoic acid (3), Oleracein E (4), Methyl-3, 4-dihydroxybenzoate (5) and Eupalitin-3-O-β-D-galactopyranoside (6)} were isolated and characterized for the first time from *B. procumbens*. Compounds (5) and (4) showed a significant antileishmanial activity with IC$_{50}$ values of 18.37 ± 0.07...
\[\mu M \text{ and } 48.19 \pm 0.06 \mu M\] while compounds (4) and (6) depicted good inhibition against acetylcholinesterase enzyme with IC\textsubscript{50} value of 54.39 \pm 0.03 \mu M and 74.07 \pm 0.06 \mu M. In Treg cell proliferation assay, Compound (4) was found most active against treg cell proliferation with percent increase (83.51) followed by compound 1 and 6 with percent increase (32.02 and 24.18), respectively. In the isolated compounds only Compound 4 showed less cytotoxicity against NIH-3T3 cell lines with IC\textsubscript{50} value of 38.81 \pm 2.93 at the test concentration of 100 \mu M. In-vivo biological activities, toxicity test of BP showed no sign of toxicity and any abnormal behavior up to a dose of 2000 mg/kg for crude extract while for compound (6) up to a dose of 500 mg/kg. The peripheral analgesic activity of crude extract of B. Procumbens at dose of 200 mg/kg exhibited significant effect (p<0.05) while at dose of 400 mg/kg showed more significant effect (p<0.01) against the antinociceptive activity. The central analgesic activity of extract (400 mg/kg) showed more significant effect (p<0.01) after 60 min of administration and remained significant till 120 min. The anti-inflammatory activity of crude extract (400 mg/kg) showed maximum percent inhibition (47.98 %) of paw edema at 5 hrs of observation. In hepatoprotective assay, compound (6) of B. Procumbens at dose of 60 mg/kg with CCl\textsubscript{4} significantly reduced (p<0.01) the levels of SGPT and SGOT (26 \pm 1.34 U/ml and 42.92 \pm 1.6 U/ml) enzymes when compared with the group treated with CCl\textsubscript{4} alone (23.85 \pm 1.72 U/ml and 34.53 \pm 2.41U/ml). Similarly, the serum levels of ALP and total bilirubin (179.22 \pm 3.41U/ml and 3.23 \pm 0.19 mg/dl) significantly decline (p < 0.01) was also observed in group treated with 60 mg/kg dose. The current research work strongly supports the ethnomedicinal properties of B. procumbens which has been used widely without scientific validation for the treatment of many diseases especially hepatic disorders. Therefore, B. Procumbens can utilizing for value added herbal medicines/products as this species grow commonly as weeds in Pakistan even under tough conditions of enviroments.
1. INTRODUCTION

1.1 General Introduction

Plants uses like nutritive, therapeutic and bio-pesticidal are developing a common trend in different parts of the world. The tendency towards plants as a medicinal agent around the globe is due to lesser side effects, better compatibility with human body (Partap et al., 2012), relatively reasonable cost and well efficiency as compared to synthetic drugs (Razak et al., 2011). WHO (World Health Organization) assessed that 4 billion people (80 % population) use herbal medicine for their primary healthcare (WHO, 2002). Furthermore, people also used herbal medicine due to lack of healthcare facilities in rural area (Govendarajan, 2001) as they cannot approach the products of the Western pharmaceutical manufacturing products (Salie et al., 1996). According to the WHO survey that traditional healers treat 90 % patients in Bangladesh, 80 % in India, 75 % in Nepal, and 65 % in Sri Lanka. In Pakistan, 60 % of the inhabitants, particularly in rural areas are getting healthcare service by conventional practitioners (Hakims), which suggests herbal medicines (Ahmed et al., 2004; Gilani et al., 2006). Plants have been rich sources of medicines since long as they produce a multitude of biologically-active compounds, most of which possibly uses in the treatment of different diseases in human and animals.

1.2 Medicinal properties of plants

Phytochemical compounds in plants are compounds delivered by plants having pharmacological or toxicological properties in man and animals. These non-nutrient (e.g. vitamins and minerals) chemical compounds are often denoted as phytochemicals or plant secondary metabolites” (Bernhoft, 2010).

1.2.1 Biosynthesis and purpose of secondary metabolites (Natural products)

Secondary metabolites are manufactured inside the plants as well the primary biosynthetic routes of compounds intended at plant growth and development, such as amino acids, proteins, lipids and carbohydrates. They can be observed as products of biochemical “side tracks” in the plant cells and not needed for normal physiological activities of the plant. Metabolites/bioactive compounds have been established as a vital
source of antimicrobial agents, herbicides, pesticides and many pharmaceutical agents. Secondary metabolites have no obvious function in primary metabolism of plant, but often have an ecological role, as assist in chemical resistance against bacteria, fungi, viruses, pollinator attractants, insects and higher predators (Karuppusamy, 2009). Furthermore, metabolites are derived from major processes such as glucose glycolysis, Krebs cycle, and photosynthesis. Numerous biosynthetic pathways, different mechanisms and reactions are involved in the synthesis of bioactive compounds (Dewick, 2002) (Figure 1.1). Different classes of phytochemical constituents are found to hold important physiological/biochemical response in the treatment of different infections in human and animals.

![Figure 1.1 Plants secondary metabolites are derived from primary metabolites (Source: Tiaz and Zeiger, 2006 with slight modification)](image-url)
1.2.2 Therapeutic importance of various classes of phytochemicals

Phytochemical Compounds with biological activity are important to drug discovery because they can be used for therapeutic purposes, which are synthesized with improved activity and less toxicity (Dinkova-Kostova et al., 2005). The major bioactive compounds have been identified in plants and estimated more than 25,000 terpenoids, 12,000 alkaloids and 8,000 phenolic compounds (Lampe, 2003). To know the contribution of the phytochemicals towards health effects, some of the important phytochemicals including alkaloids, phenolics, triterpenoids and saponins are extensively reported in the literature (Saxena et al., 2013).

1.2.2.1 Alkaloids

The basic structure of alkaloids contain heterocyclic ring with basic nitrogen atoms. Alkaloids have been reported to display diverse pharmacological activities. Alkaloids are using widely in pharmaceuticals drug, poisons and narcotics (Facchini, 2001). Alkaloids like morphine, codeine, dihydrocodeine, vinblastine and taxol are using in clinical practice as analgesics and anticancer drugs (Atanasov et al., 2015). Caffeine is also an effective alkaloid, utilized mostly to neutralize the effects of poison and as CNS stimulant (Aniszewski, 2007).

1.2.2.2 Phenolics

Medicinal plants yield various active phytochemicals and phenolics are one of the examples of such bioactive compounds. Phenolics have different chemical structures and categorized as flavonoids, tannins, stilbenes, chalcones, and coumarins. Each of these compounds demonstrates a variety of pharmacological properties such as antioxidant, antibacterial, antifungal activity, anti-inflammatory, anti-HIV, and anti-leishmanial activity (Eloff et al., 2008). Phenolics are found widely in medicinal plants and its various parts (leaves, roots, and seeds) (Balasundram et al., 2006). There various factors which affect the level of phenolics in the plant such as farming methods, different variety, cultivating conditions, maturing process, and storage conditions (Naczk and Shahidi, 2006). On the other hands phenolics substance may increase under stress environments like ultraviolet rays, infection producing microorganisms, air pollution and exposure to extreme temperatures (Tomás-Barberán and Espín, 2001).
The Shikimic acid and acetic acid pathways are the main metabolic pathways for the synthesis of phenolic and other bioactive compounds (Bravo, 1998). Phenol structure contains aromatic rings as well as one or more hydroxyl substituents.

1.2.2.3 Triterpenoids and Saponins

Terpenes are phytochemicals made up of five carbon units, known as isoprene units and organized in a systematic pattern. The complexity and size of the terpene molecule depended on the number of isoprene units. The polymeric isoprene derivative, consist of at least thousands of different structure found in a wide variety of plant species such as carotenoids, steroids, and gibberellins (Volkman, 2005). Terpenoids have biological activities of interest in pharmacological applications (Anger et al., 2007). Saponins are compounds, comprising of a triterpenoid, steroid or steroidal glycoalkaloid molecule bearing one or more sugar chains (Osbourn, 1996).

1.3 Significance of medicinal plants in drug discovery

It was generally believed in the early nineteen century that utilization of plants as medicines has involved the isolation of morphine from opium (Kingham, 2001; Samuelsson, 2004). Exploration of plants for drug discovery led to the isolation of initial drugs such as morphine, quinine, cocaine, and digitoxin. In the year 2001 and 2002, about one fourth of the best selling drugs worldwide were isolated from natural products or their derivatives (Butler, 2004). A number of new plant derived drugs have been recently introduced to the U.S. market such as paclitaxel, arteether, artemisinin and galantamine (Figure 1.2).
Figure 1.2 Structures of new plant drugs recently introduced to the market
The drug paclitaxel (1) was isolated from the bark of *Taxus brevifolia* (Pacific Yew). The trade name of this drug is Taxol® which is widely used for the treatment of breast cancer. Arteether (2) was introduced in 2000 (trade name Artemotil®) and derived from artemisinin while artemisinin drug (3) was introduced in 1987 and the trade name of this drug is artemisin®. These two drugs were isolated from the plant *Artemisia annua* and both drugs are approved for malarial disease (Newman and Cragg, 2007). Galantamine (4) (also known as galanthamine, trade name Reminyl®) was isolated from the plant *Galanthus nivalis* in the early 1950s. Galantamine is accepted for curing of Alzheimer’s disease, reducing neurological degeneration by preventing acetylcholine esterase enzyme (Heinrich and Teoh, 2004; Pirtila et al., 2004).

### 1.4 Potentials of medicinal plants in Pakistan

Pakistan has a vast biodiversity of medicinal plants among developing countries. A northern area (Gilgit-Baltistan, Azad Kashmir, and northern Khyber Pakhtunkhwa) of Pakistan has extra-ordinary collection of plants, containing phytochemical compounds with a variety of biological activities (Ali and Qaiser, 1995-2005; Cotton, 1996). The northern mountains of Pakistan host about eighty percent endemic flowering plants (Ali and Qaiser, 1986). Stewart, (1972) has been reported that almost 6000 vascular plant species and 10% of the national floras contain about 600-700 plant species. These plants are used for medicinal and aromatic purposes (Shinwari, 2010) along with herbal research. Several plant species have also been used locally in certain areas for traditional formulation which is not yet explored for extensive use (Rafiq, 1997). Furthermore, it is stated that about 250-300 plant species have been available in the herbal markets of Pakistan (Williams and Ahmed, 1999)

### 1.5 Major & important medicinal plants of Pakistan

Due to its wide-ranging climatic conditions and naturally favorable habitat, Pakistan has a good potential of biodiversity of medicinal and aromatic plants (Ahmed, 2015). After discovery of their active compounds from medicinal plants, still there are many unexplored plant species which may bring positive impact in the control of various emerging diseases (Babar and Hatcher, 2005). In this chapter, only reports the main and important medicinal plants of Pakistan are highlighted with traditional uses. These plants are *Ephedra* species and *Valeriana wallichii* (Williams and Ahmad, 1999), *Tribulus terrestris*, *Withania somnifera*, *Artemesia annua*, *Silybum marianum*, *Aloe*
vera (Khan and Mahmood, 2006), Colchicum luteum, Glycyrrhiza glabra, Commiphora wightii (Ahmed, 2015). The traditional uses of these plants are mainly in the form of crude extracts, fractions and provided many active compounds which are used against several diseases as medicines. In these plants, Artemisia, Ephedra and Valeriana are the most commercially available species in traditional medicine. The importance and their traditional uses are the following:

1.5.1 Artemisia

Artemisia (Family compositae) is a vast genus of small herbaceous plant. Artemisia genus has been composed of 280 species and found in Northern hemisphere in arid regions. About 30 species of Artemisia have been described to grow naturally in Pakistan (Stewart, 1972). These herbaceous plants financial importance to Pakistan due to medicinal value and yield essential oils as well use as fodder for livestock. In Pakistan, Artemisia plants, mainly of A. maritima has been used for the extraction of santonin drug. This drug has been used very effectively against parasitic diseases, such as those caused by helminthes (P. de Mayo, 1959). Rawalpindi and Kurram chemical enterprise has been involved in the extraction of santonin and these are one of the leading manufacturers in the world (Hussain et al., 2012)

1.5.2 Ephedra

Ephedra genus has 42 species on the world wide. In Pakistan, only nine species are found, of which five species are found in Balochistan including E. procera, E. gerardiana, E. intermedia, E. sarcocarpa and E. ciliata. Ephedrine is the major isolated alkaloid from ephedra species and contains various types of pharmacological activities. Ephedra species has been used traditionally for the treatment of respiratory tract diseases while some species are used as diet supplement for weight reduction (Ghafoor, et al., 2007).

1.5.3 Valeriana

Valeriana generally well-known in Indo-Pak as valerian and locally recognized as Mushk-e-Bala (Balchur). This plant is considered to be important plant species from business point of view and used for the curing of nervous disorders like insomnia, hysteria, depression and epilepsy. The roots and rhizomes of the numerous species of this genus are used as a sleep relief, mild tranquilizer and gastrointestinal agents (Bos et
Valerian is a popular drug of the genus *Valeriana*. Jawarish jalinus is the best herbal medicine of *V. wallichii* plant and mostly used in Pakistan for stomach and abdomen diseases (Ahmad *et al*., 2009).

### 1.6 Significance of the current study

*B. procumbens* is a wild growing weed and is used traditionally by endemic and rural people of Pakistan. The whole plant or its peculiar parts have been shown a comprehensive medicinal uses for the remedy of diabetes, inflammation, heart failure, malaria, jaundice, hepatotoxicity, and other hepatic disorders (Berghofer and Schoenlechner, 2002; Samy *et al*., 1999; Srivastava *et al*., 1998 and Khan *et al*., 2013). Due to these extra-ordinary medicinal properties of *B. procumbens* have attracted pioneers in the field of medicine to identify the phytochemical compounds involved in various pharmacological activities. Little information has been reported so far regarding isolation of compounds on this plant and needed to explore chemical strucutres of bioactive compounds from this plant. The present study was designed to evaluate the nutritional, phytochemical and pharmacological activities of *B. procumbens* for the provision of valid scientific rationale to its folk uses.

### Objectives

- To investigate the proximate, minerals and amino-acids composition of *B. procumbens* plant and its roots for nutritional values.
- To determine the qualitative and quantitative phytochemical compounds of crude extract and various solvent soluble fractions.
- To carry out isolation and purification of chemical compounds using Column chromatography and thin layer chromatography.
- To perform structure elucidation of the chemical compounds using sophisticated techniques such as UV, FT-IR, MS, $^1$H-NMR, 2D NMR and $^{13}$C-NMR.
- Evaluation of various crude extract, solvent fractions and isolated compounds using In-*vitro* and In-*vivo* bioassays
2. LITERATURE REVIEW

2.1 Evolution of indigenous plant medicine system

Health care service in ancient times involved the practice of medicinal plants and its various parts for their therapeutic purposes in the forms of crude drugs, for example, tinctures, teas and adhesive mass (Balick and Cox, 1996; Samuelsson 2004). Moreover, medicinal plants remain the dominant form of medicine in most countries. About three fourths populations of the world depend upon mainly on plants-sourced natural products to meet their daily health care requirements (Barrett and Kieffer, 2001). Ginger, cloves and coriander are generally added as fresh or dried materials to use as Carminative agent (Rao and Arora, 2004).

2.2 Family Nyctaginaceae

Nyctaginaceae commonly known as four o’clock family and contains 30 genera as well as 290 species (Willis, 1973 and Mabberely, 1987). This family broadly dispersed in tropical region of hemispheres, particularly in America. In Pakistan it is categorized by 5 genera and 11 species (Nasir, 1974).

The members of the family Nyctaginaceae are trees, herbs or shrubs. The leaves are opposite or alternate, simple and do not arise from stipule. Flowers are arranged in cymose heads. Flowers may be bisexual or unisexual depending on species. Flowers arise from bracts which are showy and petals colored in most cases while in some cases they are not very prominent. Perianth has five lobes which are fused and tubular in shape. Perianth is mostly coloured petal while the lower half for example fruit is long lived. Stamens are five, more or less alternate with lobes. The filaments are unequal and anther has two locules and dehisc (discharging seeds or pollen) in longitudinal direction. Gynoeciums are with single carpel, ovary has single chamber and single ovule. Fruit is a nut or an achene enclosed in anthocarp while seeds are with endosperm (Yasin and Nasir, 1977).

2.3 The Genus Boerhavia

*Boerhavia* are the most species-rich genera of the Nyctaginaceae with 40 species and found in the tropical to subtropical, South Europe and South United States (Fosberg,
1978; Spellenberg, 2012). Out of 40 species, 4 or 5 species are found in Pakistan (Nasir, 1974), namely Boerhavia diffusa, Boerhavia diandra, Boerhavia repens, Boerhavia rubicunda, Boerhavia procumbens. The details of these species are discussed below;

2.3.1 Boerhavia diffusa

This species grows throughout the tropical region of the African and Indian continents. B. diffusa is similar to Boerhavia coccinea, Boerhavia paniculata, and Boerhavia adscendens as shown in figure 2.1. The plants are traditionally used for the remedy of several ailments in various parts of India. The whole plant as well as different parts (root & leaves) of B. diffusa has been used for the remedy of many diseases. B. diffusa roots possess significant in-vivo activities such as anti-inflammatory (Bhalla et al., 1971), anti-nematicidal (Vijayalakshmi et al., 1979) and antibacterial (Olukoya et al., 1993). Similarly, B. diffusa leaves extracts also showed significant in-vivo activities such as hepatoprotective, antioxidant, anti-nociceptive, anti-bacterial and anti-diabetic (Dhar et al., 2011). Some other activities of B. diffusa have been reported like anti-convulsant activity (Kaur and Goel, 2011), anti-diabetic activity (Pari and Satheesh, 2004), antioxidant and hepatoprotective activity (Olaleye, et al., 2010), anti-peroxidative activity (Prathapan, et al., 2011), anti-stress activity (Desai, et al., 2011) immunomodulatory activity (Aryanmanu and Kuttan, 2009).
2.3.2 *Boerhavia repens*

*B. repens* is an important medicinal plant and a rich source of therapeutic compounds (Wabale and Petkar, 2005). *B. repens* species was found specifically in Indo-Pacific and African region (Eastward to Java, Polynesia & Hawaii) (figure 2.2). This plant generally arises in sandy areas near the coastal river, rarely growing slightly inside on roadsides and does not appear to seem to be a weed (Najam, et al., 2008). *B. repens* is traditionally used for the remedy of jaundice, constipation, fever, blood purification and viral infection (Mitra and Mukherjee, 2010).
2.3.3 *Boerhavia rubicunda*

*B. rubicunda* is distributed mainly in India, Africa, Arab and Pakistan (figure 2.3). The plant is found occasionally in dry rocky or sandy habitats. The plant has similar characteristics to *B. elegana* Choisy. Various parts of the plant are reportedly used as fodder for sheep. The leaves are traditionally used for diruretic problem while the seeds are used for edible purpose (Bamber, 1916; Stewart, 1920). Furthermore, It is widely used by indigenous tribes in the traditional cuisines of South Yemen (Boulos, 1988), which is one of a staple ingredient in the manufacture of porridges; it is eaten as supplement in the mixtures of bread and cakes which is characterized by good flavor.

![Figure 2.3 Boerhavia rubicunda](image)

2.4 Plant introduction

2.4.1 *Boerhavia Procumbens*

*B. procumbens* plant is described by Joseph Banks and William Roxburgh. This plant species is an herbaceous, tropical herb, found as a weedy in waste places of clay loam soil and the foothills at altitude up to 2200 m. The binomial nomenclature of *B. Procumbens* is given in table 2.1.
Table 2.1 Taxonomical position of *B. Procumbens*

<table>
<thead>
<tr>
<th>Systematic classification</th>
<th><em>B. procumbens</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Synonym:</td>
<td><em>Boerhavia coccinea</em> Mill.</td>
</tr>
<tr>
<td>Kingdom:</td>
<td>Plantae</td>
</tr>
<tr>
<td>Division:</td>
<td>Tracheophyta</td>
</tr>
<tr>
<td>Class:</td>
<td>Magnoliopsida</td>
</tr>
<tr>
<td>Order:</td>
<td>Caryophyllales</td>
</tr>
<tr>
<td>Family:</td>
<td>Nyctaginaceae</td>
</tr>
<tr>
<td>Genus:</td>
<td><em>Boerhavia</em></td>
</tr>
<tr>
<td>Species:</td>
<td><em>Procumbens</em></td>
</tr>
<tr>
<td>Sheet No.</td>
<td>BOT. 20077(PUP)</td>
</tr>
</tbody>
</table>

2.4.2 Vernacular names (Names in different languages)

- **English:** Spreading hogweed/ pig weed
- **Urdu:** Biskhapra
- **Punjab:** Jangli itsit
- **Sindh:** Sentori
- **Baluchistan:** Wasao

2.4.3 Morphology

*B. procumbens* is a perennial, growing along with the ground flatly having irregular branches and hairy herb. The stem is woody, position of branches is below and has red color. Leaves arrangement is opposite while both are unequal in size. The leaves are oval, oblong or somewhat heart-shaped with a notch at the base having size 1-5 × 0.3-3.8 cm. leaf boarder are wary, cuspidate (shape spiny end) or obtuse end, the undersurface is often whitish. Bracts and bracteoles are 1.5-2mm long and are long oval shaped with pointed ends while the margin is membranous. Flower is about 3 mm long, arranged in irregular axils forming panicles inflorescence. Perianth is bell shaped, pinkish in color and fall off soon (deciduous). Stamens are 2-3 in number and longer than petals. Filaments are slender shaped and arise from axil of a scale like structure. Ovary is oval shaped and about 1mm long; style is long about 1.5 mm while stigma is plate-like. Fruit is somewhat clup-shaped, up to 3 mm long having 5 ribs.
2.4.4 Geographical Distribution

It is found worldwide in indo-pak subcontinent and tropical Africa. In Pakistan it is available in various areas such as Sindh, Baluchistan, Jhelum, Multan, Swat, Malakand, Hazara, Abbottabad and Peshawar (Nasir, 1977).

![Naturally Growing plants (A)](image1) ![Leaves (B)](image2)

![Flowers and Fruits (C)](image3) ![Roots (D)](image4)

*Figure 2.4 Various parts of *B. procumbens*: *B. procumbens* plant (A), Leaves (B), Flowers & Fruits (C) and Roots (D)*
2.5 Ethnobotanical uses

A vast number of ethnomedicinal usages of several species of the genus *Boerhavia* have been acknowledged in literature. The results of comprehensive literature survey are presented in table 2.2 indicating the plants species, parts used and diseases.

**Table 2.2. Ethnobotanical uses of various parts of the genus *Boerhavia***

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Plants species</th>
<th>Parts used</th>
<th>Diseases</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td><em>B. repens</em></td>
<td>Whole plants</td>
<td>Jaundice (Purkayastha &amp; Nath, 2006), fever (Bhosale <em>et al.</em>, 2009), constipation (Mitra &amp; Mukherjee, 2010), blood purifier and anti-viral use (Bhosale <em>et al.</em>, 2009)</td>
</tr>
<tr>
<td>2.</td>
<td><em>B. paniculata</em></td>
<td>Whole plants</td>
<td>This species has been used in the remedy of kidney diseases, malaria, jaundice, spleen disorders, dropsy, as anti-hemorrhagic and particularly against urinary and uterine system inflammations (Iqbal <em>et al.</em>, 2011)</td>
</tr>
<tr>
<td>3.</td>
<td><em>B. diffusa</em></td>
<td>Whole plants &amp; roots</td>
<td>The whole plant and its roots are used traditionally for the curement of diabetes, abdominal pain, inflammation, jaundice, heart diseases, bacterial infections (Nalamolu <em>et al.</em>, 2004; Baskaran <em>et al.</em>, 2011).</td>
</tr>
<tr>
<td>4.</td>
<td><em>B. coccinea</em></td>
<td>Whole plants</td>
<td>It is used in the remedy of urinary diseases (Braga, 1960)</td>
</tr>
<tr>
<td>5.</td>
<td><em>B. elegans</em></td>
<td>Leaves &amp; roots</td>
<td>The leaves of this plant are using for the therapy of ophthalmic diseases, eye infections and pain of the joints while roots are used for diuretic problem (Najam <em>et al.</em>, 2008; Sandhu <em>et al.</em>, 2011).</td>
</tr>
<tr>
<td>6.</td>
<td><em>B. erecta</em></td>
<td>Whole plants</td>
<td>This plant is used in various kinds of diseases such as malaria, hepatic disorders, jaundice, and urine infections (Berghofer <em>et al.</em>, 2002).</td>
</tr>
<tr>
<td>7.</td>
<td><em>B. erecta</em></td>
<td>Whole plants</td>
<td>This species is used as antiparasitic, antimicrobial, antiviral, antihistaminic, antioxidant and hepatoprotective (Perumalsamy <em>et al</em>., 1999; Hilou <em>et al</em>., 2006; Guerra Ordóez <em>et al</em>., 2006 &amp; Hemalatha and Petrus, 2006)</td>
</tr>
<tr>
<td>8.</td>
<td><em>B. Procumbens</em></td>
<td>Leaves &amp; roots</td>
<td>These parts are used for the remedy of various ailments like jaundice, anaemia, asthma, stomach disorders, and sore throat, analgesic and antispasmodic. (Shah and Khan 2006; Singh, <em>et al</em>., 2002 and Khan, <em>et al</em>., 2000)</td>
</tr>
<tr>
<td>9.</td>
<td><em>B. Procumbens</em></td>
<td>Whole plants</td>
<td>Similarly the whole plants are also used traditionally for the curing of jaundice, inflammation, anemia, bronchitis, piles, asthma, night blindness, asthma and cough (Akhtar and Begum, 2009; Kayani, <em>et al</em>., 2014). Moreover it is used for the purification of blood (Immanuel and Elizabeth 2009; Padmavathy and Anbarashan, 2011) and for the treatment of anaemia (Darsini, <em>et al</em>., 2009)</td>
</tr>
</tbody>
</table>

## 2.6 Chemical Compositions

The qualitative and quantative analysis of phytochemical compounds, chemical composition and essential oil were reported in some of the whole plants and its various parts regarding genus *Boerhavia*. Based on literature survey, *Boerhavia* species can be considered as a good source of essential oil and nutritional supplements.

Bokhari, *et al*., (2015) displayed the existence of diverse classes of phytochemical compounds (flavonoids, tannins, saponins, phlobatannins, cardiac glycosides, alkaloids, terpenoids, and anthraquinones) in methanolic crude extract of *B. procumbens*. The phenolic (60.45 ± 2.1 mg/g) and flavonoid (68.05 ± 2.3 mg/g) contents has been determined in ethyl acetate and *n*-butanol fractions respectively.
Abassi et al., (2012) carried out the qualitative and quantitative screening of phytochemical compounds in various fractions of *B. procumbens*. The results showed that phenolic, flavonoides and glycoside compounds were present in large amount in CHCl₃, EtOAc and n-BuOH fraction. The highest phenolic content (77.1 ± 0.6 mg/g) was found in EtOAc, CHCl₃, and n-BuOH fractions.

Abd El-Salam et al., (2013) reported various essential oil from the roots of *B. procumbens*, including Sabinene, o-Cymene, 3-Thujanone, 1-terpinene-4-ol, Limonene oxide. Iron, zinc, nickel, chromium, cobalt, copper, lead and cadmium were determined in roots, stem and leaf with different concentrations. In this study also showed that the concentration of toxic metals was found within the permissible level.

Shinde et al., (2013) investigated species such as *B. erecta, S. cumini and G. sylvestre* for fourteen (14) different macro and micro elements. Iron, zinc and molybdenum were found in maximum concentration in the species of *B. erecta, S. cumini and G. sylvestre*. These elements play a vital role against various disease resistant properties.

Juna Beegum et al., (2014) investigated the quantities analysis of flavonoids and phenolic contents of *B. diffusa* were found to be higher (5.651 g/100 g, 2.471 g/100 g, respectively) than alkaloids (0.232 g/100 g). The values of carbohydrates (10.56 mg/gm) and protein (5.76 mg/gm) were recorded higher while the quantity of fat (1.61 mg/gm) was in a lower concentration. In case of minerals analysis, only the magnesium element was reported in higher concentration (142.9 mg/100 g) in the plant.

Puranik et al., (2012) evaluated the fresh leaves of *B. diffusa* for preliminary phytochemical analysis and detected only alkaloids, flavonoids and saponins in the leaves extract of sample plant. In the proximate analysis, the value of moisture (76.04 %) and carbohydrate (17.14 %) showed to be higher concentration then other nutrients of the sample. Similarly, the phosphrous (151.45 mg/100 g), sodium (160.21 mg/100 g), calcium (218.24 mg/100g) and magnesium contents (8.93 mg/100 g) were also reported in higher concentration as compared to other minerals content.

Ujowundu et al., (2008) carried out the phytochemical screening and detected alkaloids, flavonoids and saponins in the aqueous leaf extracts of *B. diffusa*. Moreover, the proximate and vitamins analysis of *B. diffusa* includes mainly moisture (82.22 %),
Nitrogen-free extract (10.56 %), ascorbic acid (44.80 mg/100 g dry weight), vitamin B₃ (97.00 mg/100 g) and vitamin B₂ (22.00 mg/100 g) respectively. In case of mineral analysis, sodium (162.50 mg/100g) and calcium (174.09 mg/100 g) were recorded in higher concentration then magnesium (8.68 mg/100 g) in the defatted leaf extracts of *B. diffusa*.

Gupta and Yadav, (2013) determined the concentration of various nutrients and its energy in the dried leaves of *B. diffusa*. Their investigation included moisture content (8.48 ± 0.27 g/100 g), inorganic matter (2.4 ± 0.07 g/100 g), protein constituent (1.45±0.09 g/100 g), ether extract (1.12 ± 0.00 g/100), dietary fiber (0.97 ± 0.03 g/100 g), nitrogen-free extract (5.51 ± 0.56 g/100 g) and calorific value (38 ± 1.46 kcals/100 g). The values of iron and calcium were found as 20.02 ± 0.31 mg/100 g and 250.6 ± 2.17 mg/100 g in dried leaves while ascorbic acid and β-carotene were found as 38.0 mg/100 g and 44.0 mg/100 g respectively.

Ammar *et al.*, (2014) reported that the phenolic (253.9 ± 0.9 mg/g) and flavonoids compounds (23.68 ± 0.6 mg/ g) were contained highest in the seeds of *B. elegana*.

Al-Farga *et al.*, (2015) investigated *B. elegana* for oil (11.49 %), organic matter (6.88 %), moisture (6.12 %), protein content (14.60 %), nitrogen-free extract (24.77 %) and dietary fiber (36.13 %). The ether extracted oil from the leaves of *B. elegana* contained a high quantity of oleic (57.77 %), palmitic (18.65 %) and linoleic (12.88 %) acids.

### 2.7 The techniques used for plant-based drug discovery

In the modern days, investigation of the phytochemical compounds contained in the traditionally-acknowledged medicinal plants has established significant interest in drug research and developmental projects. Several advanced techniques are presently available, especially in separation, spectroscopic and bioassay procedures.

The approach of natural product isolation mainly from plants has transfered from “old-fashioned approach” established on a straight forward-crude-extraction, to the current day ‘bioassay-guided isolation’ technique (figure 2.5). The old strategy phytochemical techniques related to chemicals synthesized by plants, hence the main focus in that case was on chemical structure, metabolism/biosynthesis, distribution and bioactivity of
these substances. Although both methodologies rely upon ethnopharmacological information, focus of the newer methodology is upon bioactivity guided identification and isolation of lead compounds (Seidel, 2006). Furthermore, bioassay guided isolation involves successive extraction, isolation and purification steps with repeated bioactivity testing before moving to the next step. This approach has been applied to several drug discovery studies aimed at plant-derived natural products (Sudha and Srinivasan, 2014, Rashid et al., 2014).

![Bioassay-guided natural product discovery process](image)

**Figure 2.5** Bioassay-guided natural product discovery process

After the identification of the chemical structure of the isolated compounds, the next step is to conclude the underlying mechanism responsible for the pharmacological effects. There are numerous methods for determining the pharmacological-mechanism of newly discovered drugs, involving proteomics and genomics studies (Oberg et al., 2011). Proteomic techniques can recognize alterations in proteins as potential drug targets, and this can also help understand a drug’s mode of action. Proteomics can also identify post-translational protein modifications such as phosphorylation, glycosylation, acetylation, and proteolysis, and sequence variants such as mutants, alternatively spliced isoforms, and amino acid polymorphisms (Mann and Jensen, 2003; Zhang, 2011). After investigation the mechanism of action, the next steps is *in-vivo* bioassay
and toxicological studies followed by clinical trials and pharmaceutical production process (Sarker et al., 2006)

The new strategy for the isolation of phytochemical compounds from natural products utilizes early bioactivity screening of the crude extracts. The extraction (maceration method), fractionation (using different organic solvents), isolation (column chromatography), and purification of the bioactive compounds are driven by in-vitro bioassay, with pharmacologically-active fraction only entering the next phase of the process (Sarker et al., 2006). The following considerations are necessary for successful isolation and identification of unknown compounds from plant sources.

2.7.1 Extraction methods

The extraction of chemical constituents from plant source mainly depends on the selection of extraction process (Smith, 2003; Sasidharan et al., 2011). The biologically active portion of plant has been separated by standard procedures using organic solvents (Handa et al., 2008). Various extraction methods have been used for the extraction of plant materials, but the most common ones are the maceration technique. Vongsak et al., (2013) reported that maceration is a more valid, economical, (Seidel, 2006 & Handa, 2008) and convenient method for thermolabile for drug as well as small and medium enterprises then other extraction methods. However, this method requires more solvent and produces a limited yield of compounds (Dhanani et al., 2013). Moreover, the maceration method is also time consuming, requiring time frames from 2 to 10 days (Cunha et al., 2004; Woisky and Salatino, 1998).

2.7.2 Solvent-solvent fractionation

This method is also known as partitioning. The main objective of solvent-solvent partitioning is to simplify extraction by fractionating the chemical compounds into broad groups based on their solubility (Suffness and Dous, 1982). Solvent-solvent extraction is a basic method which is mainly performed in separating funnel (Hu, 1999). This method is the most well-known since even the smallest quantity of the substance can be easily extracted. Kupchan, (1969) reported that this process play a major role in the discovery of many new compounds which were impossible to isolate with other classical extraction approaches. Both polar and non-polar compounds can be
separated easily using this method. Sarkar et al., (2006) explained the disadvantage of this method includes emulsion formation, time consuming, and requirement of large volume of solvents.

2.7.3 Choice of organic solvents

Effective exploration of pharmacologically active compounds from plant material is mainly dependent on the types of organic solvent used in the extraction method. The selection of solvent and polarity of the targeted compound is the most important factor for extraction (Das et al., 2010). In addition, these factors also consider important for effective extraction such as affinity between solvent and solute molecules, use of co-solvent, low toxicity and ease of evaporation at low heat (Eloff et al., 1998). Commonly nonpolar (chloroform, n-hexane) and polar (methanol, ethanol, acetone, ethyl acetate) organic solvents are both used for extraction (Fuleki et al., 1997; Jayaprakasha et al., 2003; Ozkan et al., 2004; Xu and Chang, 2007). The combinations of these solvents often with different proportions of water have been used for the extraction of phenolic compounds from plant-derived materials. Meanwhile, nearly all of the known antibacterial and antifungal compounds from plants are aromatic hydrocarbons and obtained via ethanol or methanol extraction (Serkedjieva and Manolova, 1992). Especially, methanol and acetone solvents are usually more effective for the extraction of lower molecular weight compounds like polyphenols and higher molecular weight compounds like flavanols (Metivier et al., 1980; Labarbe 1999; Guyot et al., 2001; Prior et al., 2001). Brain & Turner, 1975; Vanwyk & Wink 2004; Shi et al., 2005 reported that ethanol is nontoxic solvent for human consumption and considered suitable for the extraction of polyphenols. The combined application of water and ethanol could also improve the extraction rate, but too high water content would increase concomitant extraction of other compounds. Polar solvents are used for the extraction of hydrophilic compounds while nonpolar solvent is used for the removal of chlorophyll (Gomez et al., 1996; Baydar et al., 2006; Lafka et al., 2007). Other nonpolar solvents such as Chloroform were found to be the best for the extraction of non-polar bioactive compounds (Harmala et al., 1992).
2.7.4 Chromatographic techniques

Bioactive compounds are often found in crude extract in the form of mixture with similar structures and polarities. Chromatography associated technologies are the most regularly used methods for isolation of desired compounds from the mixture. The next step is the purification of the isolated compounds from the crude extract and it is an essential task as purified compounds are more active than the impure compounds (Rodrigues et al., 1999). There are various Chromatographic separation techniques involve in the isolation and purification of these bioactive compounds (Sasidharan et al., 2011).

2.7.4.1 Column chromatography

Column chromatography is an effective method for the isolation of targeted compounds from a mixture and principally based on colour bands (Sasongko et al., 2011; Kenkel, 2003). Çitoğlu and Acıkara, (2012) have used this technique for fractionation of monoterpenoid compounds. Sur, (1991) reported the two elution systems of isocratic and gradient elutions with combinations of different solvents for the continuous isolation of bioactive compounds on the basis of increasing polarity. Johnson and Stevenson, (1977) carried out open column chromatography using glass column where the solute adsorption depends upon silica gel as polar adsorbent. Another study was conducted by Espinoza et al., (2008) on column chromatography using hexane-ethyl acetate gradient as the eluents for purification of Idriella sp. extract. Although Flash chromatography is time-consuming and gives poor recovery but still used for the separation of phytochemical constituents from plant crude extracts (Ikan, 1991). Wang and co-worker isolated and purified succinic acid from dried rhizome of Houttuynia cordata plant by column chromatography on silica gel and Sephadex LH-20 (Wang et al., 2007).

2.7.4.2 Thin layer chromatography and HP-TLC

Thin layer chromatography (TLC) or High performance Thin layer chromatography (HPTLC) is mostly used as an economical technique for the separation and identification of samples. TLC is therefore often expressed as a preliminary method for HPLC (Rozylo and Janicka, 1991; Rozylo and and Janicka, 1996). TLC and HPTLC
techniques can be used for qualitative and quantitative analysis in the fields of medicine, pharmaceuticals, and food analysis (Weins and Hauck, 1996; Kalász and Bátorni, 1997). It has been reported that TLC is a dominant tool to explore unknown materials in bulk drugs (Szepesi and Nyiredy, 1996). Various drugs (ethinyl estradiol, cyproterone, alfuzosin, tramadol and pentazocine) have been determined quantitatively by HPTLC method (Pavic et al., 2003 and Fayed et al., 2006). TLC has been employed for the determination of some steroids, pioglitazone, celecoxib and noscapine (Cimpoiu et al., 2006; Gumieniczek et al., 2004; Bebawy et al., 2002 and Ashour et al., 2009). De Oliveira et al., (2012) also utilized silica gel TLC for the isolation of phenolic compounds from *Baccharis trimera* extract. Seanego and Ndip (2012) have used TLC for the isolation of bioactive molecules from *Garcinia kola* seeds; Zeeshan et al., (2012) from leaves of *Ageratum houstonianum*; Lawrence et al., (2009) from leaves of *Aloe vera*. Similarly, Kumar et al., (2010) using solvent system of chloroform and methanol at ratio of 95:5 on aluminium plates of TLC for the preliminary screening of extract of root and aerial parts of *Andrographis serpyllifolia* extract.

### 2.7.4.3 High performance liquid chromatography (HPLC)

Another important purification technique is the HPLC and used widely for the quantitative analysis as well as isolation of natural products (Cannell, 1998). This technique is considered to be non-destructive and can be useful for thermally unstable compounds. This method is achieving popularity amongst numerous analytical methods as the main choice for fingerprinting study in the quality control of herbal plants (Fan et al., 2006). Some scientist described that HPLC can be used for the characterization and quantification of bioactive compound from the crude extracts such as phenolic compounds, steroids, flavonoids, alkaloids (Boligon *et al.*, 2012; Barbosa Filho *et al.*, 2014 and Reis Ede *et al.*, 2014). Rochfort *et al.*, (2006) reported the isolation and purification of glucoraphanin from broccoli by preparative HPLC. Asghari *et al.*, (2011); Deng *et al.*, (2012); Nazzaro *et al.*, (2012) isolated various bioactive compounds from cinnamom husks, fruit wastes, chestnut and hazelnut shells using HPLC.
2.7.4.4 Gas liquid chromatography

It is also a powerful technique to detect volatile organic compounds. Various organic compounds have been determined using this technique such as isotretinoin, cocaine, and betamethasone valerate (Lima et al., 2005; Zuo et al., 2004 and Somuramasami et al., 2011). Gas chromatography has been used for the analysis of impurities in pharmaceutical products (Watson, 1999). Moreover, some other methods have been employed to identify bioactive compounds amongst are the liquid chromatography (LC) and gas chromatography (GC) coupled with mass spectrometry (Cai et al., 2002). LC-MS technique is mainly used for quality control and assurance within the pharmaceutical industry (Ermer, 1998; Nicolas and Scholz, 1998). This technique delivers plentiful information for structural elucidation of the compounds when tandem mass spectrometry (MS) is applied (Ye et al., 2007). GC/MS and LC/MS have been used for the analysis of bioactive compound from pomegranate waste and betel leaf (Anand, 2005; Nalina and Rahim, 2007; Dwivedi et al., 2010; Sugumaran et al., 2011).

2.7.5 Nuclear magnetic resonance spectroscopy (NMR)

NMR is a vital instrument in the innovation of natural products mostly changing all traditional chemical degradation techniques that were used for structural characterization (Mahrous and Farag, 2015). NMR technique has been used for the screening and composition of drugs and drug products in pharmaceutical formulations and biological fluids (Shuker et al., 1996; Salem et al., 2006 and Reinscheid, 2006). In the past few years, NMR found a widespread application in quantitative analysis in order to determine the impurity of the drug (Mistry et al., 1999). In addition to NMR, Fourier transform-Infra red (FT-IR) has also proven to be a valuable tool to determine the functional groups present in an unknown sample (Eberhardt et al., 2007). The identification of functional groups in a compound may be detected using IR by analyzing the different bonds present. These bonds have different vibrational frequencies and these frequencies in the form of absorption band in the infrared spectrum (Kemp, 1991b).
2.7.6 Mass spectrometry (MS)

MS is an analytical method and used to determine the molecular weights, elemental composition and structure of compounds in unknown mixture by matching their spectra with reference spectra (Patil and Wanjare, 2017). Alborn and Stenhagen, (1987) determined the molecular mass of various compounds such as caffeic, o-coumaric and sinapic acid using electron impact ionization (EI). The Information related molecular mass and sugar sequence by cleavage of glycosidic bonds has been determined in saponins using Fast atom bombardment (FAB) (Ogata, 2008; Nichenametla et al., 2006; Seelinger et al., 2008 and Mayer et al., 2008). The structural characterizations of polar and thermally labile molecules such as polypeptides, carbohydrates and natural glycosides have been determined using Electrospray ionization (Ghosh and Konishi, 2007; Srinivas et al., 2007; Tanaka et al., 1993).

2.8 Isolated compounds from genus Boerhavia

Literature review on the genus Boerhavia showed that a number of biologically active compounds have been isolated from various species of this genus. Hence, the genus Boerhavia is a potential source of different classes of phytochemical compounds such as flavonoids, Cardiac Gylcosides, phenols, triterpenoids and steroids. The list of phytochemical compounds is presented in Table 2.4.
<table>
<thead>
<tr>
<th>S.No.</th>
<th>Compound names</th>
<th>Source</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Flavone</td>
<td><em>B. procumbens</em></td>
<td>Prajapati <em>et al.</em>, 2006</td>
</tr>
<tr>
<td>2</td>
<td>Glucose</td>
<td><em>B. procumbens</em></td>
<td>Prajapati <em>et al.</em>, 2006</td>
</tr>
<tr>
<td>3</td>
<td>Sucrose</td>
<td><em>B. procumbens</em></td>
<td>Prajapati <em>et al.</em>, 2006</td>
</tr>
<tr>
<td>4</td>
<td>Myricetin</td>
<td><em>B. procumbens</em></td>
<td>Bokhari <em>et al.</em>, 2015b</td>
</tr>
<tr>
<td>5</td>
<td>Rutin</td>
<td><em>B. procumbens</em></td>
<td>Bokhari <em>et al.</em>, 2015b</td>
</tr>
<tr>
<td>6</td>
<td>Caffeic acid</td>
<td><em>B. procumbens</em></td>
<td>Bokhari <em>et al.</em>, 2015b</td>
</tr>
<tr>
<td>7</td>
<td>Coccineone A</td>
<td><em>B. coccinea</em></td>
<td>Messana <em>et al.</em>, 1986</td>
</tr>
<tr>
<td>8</td>
<td>Coccineone B</td>
<td><em>B. coccinea</em></td>
<td>--do--</td>
</tr>
<tr>
<td>9</td>
<td>9-O-methyl-10-hydroxycoccineone B</td>
<td><em>B. coccinea</em></td>
<td>--do--</td>
</tr>
<tr>
<td>10</td>
<td>Coccineone C</td>
<td><em>B. coccinea</em></td>
<td>--do--</td>
</tr>
<tr>
<td>11</td>
<td>Coccineone D</td>
<td><em>B. coccinea</em></td>
<td>Messana <em>et al.</em>, 1986</td>
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<tr>
<td>12</td>
<td>Coccineone E</td>
<td><em>B. coccinea</em></td>
<td>Messana <em>et al.</em>, 1986</td>
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<tr>
<td>13</td>
<td>Boeravinone A</td>
<td><em>B. diffusa</em></td>
<td>Kadota <em>et al.</em>, 1989</td>
</tr>
<tr>
<td>14</td>
<td>Boeravinone B</td>
<td><em>B. diffusa</em></td>
<td>Lami <em>et al.</em>, 1990</td>
</tr>
<tr>
<td>16</td>
<td>10-demethylboeravinone C</td>
<td><em>B. diffusa</em></td>
<td>Lami <em>et al.</em>, 1990</td>
</tr>
<tr>
<td>17</td>
<td>Boeravinone D</td>
<td>--do--</td>
<td></td>
</tr>
<tr>
<td>18</td>
<td>Boeravinone E</td>
<td>--do--</td>
<td></td>
</tr>
<tr>
<td>19</td>
<td>Boeravinone F</td>
<td><em>B. diffusa</em></td>
<td>Lami <em>et al.</em>, 1991</td>
</tr>
<tr>
<td>20</td>
<td>Boeravinone G</td>
<td><em>B. diffusa</em></td>
<td>Borrelli <em>et al.</em>, 2005</td>
</tr>
<tr>
<td>21</td>
<td>Boeravinone H</td>
<td><em>B. diffusa</em></td>
<td>Borrelli <em>et al.</em>, 2005</td>
</tr>
<tr>
<td>22</td>
<td>6-O-demethyl boeravinone H</td>
<td><em>B. diffusa</em></td>
<td>Borrelli <em>et al.</em>, 2005</td>
</tr>
<tr>
<td>23</td>
<td>Boeravinone I</td>
<td><em>B. diffusa</em></td>
<td>Ahmed-Belkacem <em>et al.</em>, 2007</td>
</tr>
<tr>
<td>25</td>
<td>Ursolic acid</td>
<td><em>B. diffusa</em></td>
<td>Lami <em>et al.</em>, 1991</td>
</tr>
<tr>
<td>26</td>
<td>Hypoxanthine-9-L-arabinofuranoside</td>
<td><em>B. diffusa</em></td>
<td>Sahu <em>et al.</em>, 2008</td>
</tr>
<tr>
<td>27</td>
<td>2,4,5-Trihydroxydiphenyl methane</td>
<td><em>B. diffusa</em></td>
<td>Jain &amp; Khanna, 1989</td>
</tr>
<tr>
<td></td>
<td>Compound</td>
<td>Genus</td>
<td>References</td>
</tr>
<tr>
<td>---</td>
<td>-----------------------------------------------</td>
<td>---------</td>
<td>--------------------</td>
</tr>
<tr>
<td>28</td>
<td>Liriodendrin</td>
<td><em>B. diffusa</em></td>
<td>Lami et al., 1991</td>
</tr>
<tr>
<td>29</td>
<td>Aglycone A</td>
<td><em>B. diffusa</em></td>
<td>Jain &amp; Khanna, 1989</td>
</tr>
<tr>
<td>30</td>
<td>Aglycone B</td>
<td><em>B. diffusa</em></td>
<td>Jain &amp; Khanna, 1989</td>
</tr>
<tr>
<td>31</td>
<td>1-glucopyrano-2-benzyl-4,5-dihydroxybenzene</td>
<td><em>B. diffusa</em></td>
<td>Jain &amp; Khanna, 1989</td>
</tr>
<tr>
<td>32</td>
<td>Boerhavisterol (9,10-Secostigmasta-5,8-dien-3-ol)</td>
<td><em>B. diffusa</em></td>
<td>Gupta &amp; Ali, 1998</td>
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<tr>
<td>33</td>
<td>Indole</td>
<td><em>B. diffusa</em></td>
<td>Pereira et al., 2009</td>
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<td>34</td>
<td>Eugenol</td>
<td><em>B. diffusa</em></td>
<td>Pereira et al., 2009</td>
</tr>
<tr>
<td>35</td>
<td>Menthol</td>
<td><em>B. diffusa</em></td>
<td>Pereira et al., 2009</td>
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<tr>
<td>36</td>
<td>Isorhamnetin</td>
<td><em>B. erecta</em></td>
<td>Stintzing et al., 2004</td>
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<tr>
<td>37</td>
<td>Kaempferol</td>
<td><em>B. erecta</em></td>
<td>Stintzing et al., 2004</td>
</tr>
<tr>
<td>38</td>
<td>Quercetin</td>
<td><em>B. erecta</em></td>
<td>-do-</td>
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<tr>
<td>39</td>
<td>Catechin</td>
<td><em>B. erecta</em></td>
<td>-do-</td>
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<td>40</td>
<td>Betanin</td>
<td><em>B. erecta</em></td>
<td>Stintzing et al., 2004</td>
</tr>
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<td>41</td>
<td>Procyanidin</td>
<td><em>B. erecta</em></td>
<td>Stintzing et al., 2004</td>
</tr>
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<td>42</td>
<td>2,6-dimethoxy Benzoquinone</td>
<td><em>B. erecta</em></td>
<td>Lien et al., 2011</td>
</tr>
<tr>
<td>43</td>
<td>(+)-catechin</td>
<td><em>B. erecta</em></td>
<td>Lien et al., 2011</td>
</tr>
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<td>44</td>
<td>Eupalitin-3-O-β-D-galactopyranosyl-(1→2)-β-D-glucopyranoside</td>
<td><em>B. repens</em></td>
<td>Li et al., 1996</td>
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<td>Eupalitin-3-O-β-D-galactopyranoside</td>
<td><em>B. repens</em></td>
<td>Li et al., 1996</td>
</tr>
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<td>6-methoxykaempferol-3-O-β-D-(1→6)-Robinioside</td>
<td><em>B. repens</em></td>
<td>Li et al., 1996</td>
</tr>
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<td>47</td>
<td>Cholest-5-en-3β-ol</td>
<td><em>B. diffusa</em></td>
<td>Miralles et al., 1988</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>B. erecta</em></td>
<td>Miralles et al., 1988</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>B. repens</em></td>
<td>Miralles et al., 1988</td>
</tr>
<tr>
<td>48</td>
<td>Campesterol (24-methyl-cholest-5-en-3β-ol)</td>
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<td>Miralles et al., 1988</td>
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<td></td>
<td></td>
<td><em>B. erecta</em></td>
<td>Miralles et al., 1988</td>
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<td><em>B. repens</em></td>
<td>Miralles et al., 1988</td>
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<tr>
<td>49</td>
<td>Stigmasterol (24-ethyl-cholest-5-22(E)-dien-3β-ol)</td>
<td><em>B. diffusa</em></td>
<td>Miralles et al., 1988</td>
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<td><em>B. repens</em></td>
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<tr>
<td>50</td>
<td>Citosterol (24-ethylcholesta-5-en-3β-ol)</td>
<td><em>B. diffusa</em></td>
<td>Miralles et al., 1988</td>
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<td><em>B. repens</em></td>
<td>Miralles et al., 1988</td>
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See appendix 7: chemical structure of the isolated compounds from genus *Boerhavia*
2.9  *In-vitro* Biological activities

In the present research work, the following *in-vitro* activities were used for the evaluation of crude extract, fractions as well as isolated compounds of *B. procumbens*. Some of the activities are reported below;

2.9.1 Antimicrobial activity

Some antibacterial and antifungal studies of the methanolic extract and its solvent fractions have been reported on the genus *Boerhavia* in the literature. Those antimicrobial activities belonging to *Boerhavia* genus are summarized below;


Agrawal *et al.*, (2003) investigated the ethyl acetate extracts of aerial parts and roots of *B. diffusa* against *M. gypseum, M. fulvum* and *M. canis* using broth dilution method. The strains such as *M. gypseum* (78.83 %) followed by *M. fulvum* (62.33 %) showed maximum zone of inhibition.

Rahman *et al.*, (2014) screened the crude methanolic extract of *B. repens* against *S. aureus*, *B. subtilis*, *B. cereus*, *E. coli*, *V. cholera*, *S. typhimurium* as well as Fungal strains such as *C. albicans* and *A. brasiliensis* using disc diffusion method. The extract showed mild activity against *S. aureus* (12.17mm) while moderate activity showed against *S. typhimurium* (17.12 mm) and *C. albicans* (16.77mm).

Sheila *et al.*, (2013) evaluated methanolic extract of leaf and root of *B. coccinea* against *B. anthracis*, *S. pyogens*, *V. cholera*, *S. dysentriae*, *C. albicans* and *C. neoformans*. Moderate inhibitory activity was found against *C. neoformans* (MIC value of 0.625 mg/ml) and *C. albicans* (MIC value of 1.250 mg/ml) by leaf and root extracts, respectively. Both extract exhibited weak activity against *B. anthracis* whereas, *S. pyogens*, *V. cholera*, *S. dysentriae* were resistant at the maximum concentration of 5 mg/ml.
Dey et al., (2012) screened various solvents fractions of *B. repens* using disc diffusion method. Results showed that the CC14 and Pet. ether fractions showed mild antimicrobial activity.

Gautam et al., (2016) tested the n-hexane, ethyl acetate (EtOAc) and crude extracts of *B. diffusa* against *S. aureus, B. cereus, S. typhi* and *E. coli*. EtOAc extract showed no activity against *E. coli* whereas *S. aureus* (14 mm) and *S. Typhi* (18 mm) were resistant against crude extract and hexane extract.

Baskaran et al., (2011) observed the crude extracts of *B. diffusa* against *S. aureus, B. cereus, M. luteus* (Gram positive bacteria), *E. coli, P. aeruginosa, K. pneumonia* (Gram-negative bacteria) and fungal strains like *A. flavus, A. Niger, and C. albicans*. The ethanol extract showed strong activity against *S. aureus* (11 mm) and *E. coli* (9 mm).

### 2.9.2 Antioxidant agents

The antioxidant potential of extracts and its subsequent solvents fractions of various species of the genus *Boerhavia* have been reported in the literature. Some of the reported antioxidant activities using DPPH model are mentioned below:

Abbasi et al., (2012) reported that ethyl acetate fraction (EtOAc) of *B. procumbens* has maximum antioxidant potential (82.54 % inhibition of DPPH free radical) at the dose of 125 μg/ml (IC50 value: 37.11μg/ml) as compared to the standard butylated hydroxytoluene (IC50 value: 12.12 μg/mL).

Bokhari et al., (2015) determined the antioxidant potential of various solvent extracts of *B. procumbens* using DPPH free radical scavenging model. Significant antioxidant potential was displayed by methanol extract (IC50: 78.3 μg/ml) and ethyl acetate extract (IC50: 93.3 μg/ml) when compared to the standards ascorbic acid and rutin (IC50: 20 μg/ml & 18 μg/ml respectively)

Khalid et al., (2011) showed that the ethanolic extract of *B. diffusa* had a maximum inhibition of DPPH free radical (91.25%) at concentration of 1.50 mg/ml with IC50 value of 0.13 mg/ml.
Patel et al., (2014) screened the hydro-alcoholic extract of *B. diffusa* using DPPH model. The maximum % inhibition of extract was 80 % while the IC\textsubscript{50} value was 100 μg/ml.

Ammar et al., (2014) showed that ethanolic seed extract of *B. elegana* to be effectively inhibiting the DPPH free radical. The IC\textsubscript{50} value of the sample plant extract was 2.42 μg/ml when compared to ascorbic acid (1.47 μg/ml).

Malhotra et al., (2012) demonstrated the methanolic extract of *B. diffusa* to be strong & effective antioxidant when compared with aqueous extract of the same plant, using DPPH model.

Bhardwaj et al., (2014) compared the antioxidant capacity of leaves, stem and root extract of *B. diffusa* using DPPH model. The highest radical scavenging activity was observed in stem extract (IC\textsubscript{50}: 90.8 μg/ml) as compared to other extracts.

Dey et al., (2012) reported strong radical scavenging activity of ethyl acetate (IC\textsubscript{50}: 4.61 μg/ml) and dichloromethane fractions (IC\textsubscript{50}: 7.67 μg/ml) of *B. repens* using DPPH model. The butyl hydroxyl toluene (BHT) was used as a positive control in the study.

### 2.9.3 Brine shrimp lethality bioassay

A very limited number of studies are reported on the cytotoxic activity of crude extract and its subsequent solvents fractions of *Boerhavia* genus in the literature. Here, a few studies are described;

Dey et al., (2012) evaluated petroleum ether fraction and ethyl acetate fraction of *B. repens*, which showed promising cytotoxic potential with LC\textsubscript{50} values of 2.24 μg/ml and 3.57 μg/ml.

Rahman et al., (2014) assessed the LC\textsubscript{50} value (4.152 μg/ml) and LC\textsubscript{90} value (124.33 μg/ml) of the crude methanol extract of *B. repens* using brine-shrimp lethality assay in comparison with positive control, vincristine sulphate, a potent cytotoxic agent.

Gautam et al., (2016) carried out the brine shrimp lethality assay of methanolic extract of *B. diffusa* and determined the LC\textsubscript{50} value of the sample to be 165.19 μg/mL.
Apu et al., (2012) tested the n-hexane extract of *B. diffusa* for cytotoxicity. The results showed negligible cytotoxic potential (LC$_{50}$: 140.55 μg/mL) of *B. diffusa* when compared to Potassium dichromate.

Adoum, (2009) reported significant cytotoxic potential of crude extract of *B. diffusa* against brine shrimp larvae having LC$_{50}$ values less than 60 μg/ml.

**2.9.4 Other in-vitro activities**

The other in-vitro assays includes in the present study such as;

- Larvicidal activity (*C. quinquefasciatus*)
- Phytotoxic assay (*Lemna minor*)
- Anti-leishmanicidal assay
- Acetyl cholinesterase (AChE) inhibition assay
- Cytotoxic activity (NIH 3T3 cell lines)
- Treg cell proliferation assay

These bioassays have been used to evaluate the crude extract, solvents fractions and isolated compounds of *B. Procumbens*. However, this plant had not been explored so far, therefore no published scientific evidence on these assays. So this is the first study to report these activities from *B. Procumbens* plant.

**2.10 In-vivo biological activities**

**2.10.1 Toxicological studies**

Some toxicological studies have been carried out on the genus of *Boerhavia*. Hiruma-Lima et al., (2000) investigated the extract of lyophilized decoction and juice of fresh leaves of *B. diffusa* for acute oral toxicity. No indication of toxicity was seen up to a dose range of 5000 mg/kg of body weight in mice and also no body or organ weight gain was observed.

Dhar et al., (1968) examined the root extract of *B. diffusa* at a dose of 1g/kg of body wt remained the maximum tolerated dose for mice and showed no adverse effect and mortality in mice.
Chandan et al., (1991) evaluated the alcoholic extract of *B. diffusa* for hepatotoxicity in mice. The extract shows no signs of toxicity at an oral dose of 2 g/kg body wt.

Sharma et al., (2001) studied the alcoholic leaves extract of *B. diffusa* for acute toxicity and observed no signs of toxicity at the dose of 2 gm/kg while aqueous extract exhibited renal tubular toxicity and myocardial degeneration in mice.

Venkatesh et al., (2013) revealed the safety of aqueous extract of *B. diffusa* in term of mortality and behavioral changes when administered orally at a dose of 5g/kg body weight.

### 2.10.2 Anti-nociceptive activity

Hiruma-Lima et al., (2000) using acetic acid induced abdominal writhing and hot plate model to determine the analgesic effect of lyophilized decoction and fresh leaf juice of *B. diffusa*. The leaf juice and lyophilized decoction are both used at a dose (p.o) of 1000 mg/kg. The fresh leaf juice produced a significant increase in latency while the lyophilized decoction, however, only raised the pain thresholds during the first 30 min of observation in the hot-plate model.

Shubha and Govindaraju, (2013) assessed the crude extracts in different solvents (petroleum ether, dichloromethane, ethanol and water) of *B. diffusa* for analgesic activity. The ethanol extract at a dose of 200 mg/kg body weight was found to be the most active extract against acetic acid induced writhing as compared to the standard drug analgin (Metamizole sodium).

### 2.10.3 Anti-inflammatory activity

Bokhari et al., (2015) used the of *n*-butanol and methanol extracts of *B. procumbens* against carrageenan-induced paw edema. The results revealed that these extracts significantly inhibited the edema after 1st, 2nd, and 3rd hour as comparable to standard drug diclofenac potassium.

Hiruma-Lima et al., (2000) evaluated the lyophilized decoction and fresh leaf juice of *B. diffusa* against carrageenan induced edema in mice. They found that neither
lyophilized decoction nor fresh leaf juice dose (1000 mg/kg) significantly decreased paw swelling compared to the reference drug indomethacin.

Oladele et al., (2011) investigated aqueous root extract of *B. diffusa* against Carrageenan-induced paw edema in rats. The aqueous extract at doses of 100-400 mg/kg significantly inhibited (p<0.05) carrageenan-induced paw edema in a dose dependent manner.

Bhalla et al., (1971) exhibited anti-inflammatory effect of *B. diffusa* leaf extract against carrageen induced rat paw edema models. The results showed that this plant has good anti-inflammatory properties.

Nagarajaiah et al., (2013) evaluated the aqueous root extract at dose of 1000 mg/kg of *B. diffusa* and compared to the standard drug (valdecoxib) at dose of 5 mg/kg. The extract significantly (P <0.001) inhibited the carrageenan induced rat paw edema when compared valdecoxib. The percent inhibitory effect of extract and valdecoxib was 52.67 % and 82.6 7% respectively after 5 hrs of observation.

Bairwa et al., (2013) isolated boeravinone from the methanolic root extract of *B. diffusa* and tested against carrageenan-induced paw edema model. This compound exhibited significant effect (56.6 % inhibition of edema at 50 mg/kg), which was better than the positive control in rats.

Muthu et al., (2014) studied the ethanol extract of *B. diffusa* at the concentration 400 mg/kg body weight and showed significant anti-inflammatory effect up to 240 min and indomethacin was used as standard drug in this study.

### 2.10.4 Hepatoprotective activity

Chakraborty and Handa, (1989) reported hepatoprotective activity of the isolated steroidal and flavone compounds (androst 5-ene analogue and 6, 5’-dimethoxy- 5, 7, 3’-trihydroxyflavone at concentration of 200 μg/ml & 50 μg/ml, respectively) from the aerial parts of *B. diffusa* against CCl₄ intoxication models.

Rawat et al., (1997) observed the protective effect of aqueous extract (2 ml/kg) and powdered form (150 mg/kg) of the roots of *B. diffusa* in thioacetamide intoxicated in
albino rats. This study proved that aqueous form of sample was more protective effect than the powder form.

Chandan et al., (1991) demonstrated the protective effect of alcoholic extract of B. diffusa against CCl₄ induced intoxication in rats and mice.

Jayavelu et al., (2013) showed protective effect in methanol extract of root and aerial part (500 mg/kg. b. wt.) of B. diffusa in Ibuprofen-induced hepatotoxicity in albino rats. Results showed that the root possesses more hepatoprotective efficacy than the aerial part of B. diffusa.

Venkatesh et al., (2000) studied hepatoprotective effect of crude extract of stem & leaves of B. diffusa in CCl₄ induced toxicity in albino rats. Results showed significant protective effect of the extract in terms of liver enzymes in CCl₄ treated rats like SGOT, SGPT, SALP, and Total Bilirubin.

Olaleye et al., (2010) used aqueous and ethanolic leaves extracts of B. diffusa against acetaminophen-induced toxicity in rats and results revealed that these two extracts effectively decreased the levels of alkaline phosphatase, aspartate aminotransferase, and bilirubin in the serum.
3. MATERIALS & METHODS

3.1 Experimental conditions

Plant drying, chopping and grinding were conducted at pharmaceutical pilot plant while extraction and fractionation were carried out at food microbiology section of PCSIR Laboratories complex, Peshawar, Pakistan. The pharmacological activities \((in-vivo)\) were carried out in Animal House and Pharmacognosy Department, University of Karachi. Biological activities \((in-vitro)\) were conducted at Department of Agricultural Chemistry, University of Agriculture, Peshawar. Compounds isolation, NMR spectroscopic and spectrometric studies as well as some special \(in-vitro\) activities were performed in HEJ, Research Institute of Chemistry, University of Karachi, Pakistan.

3.2 Collection of Plant Materials

The studied plant \((B. procumbens)\) was collected in the month of June from botanical garden of PCSIR Laboratories Complex Peshawar, Pakistan. The voucher specimen was deposited (Bot. 20077) in the herbarium of Botany Department, University of Peshawar, Pakistan for future reference (Figure 1).
3.3 Extraction of plant materials

The sample plant washed with running water and dried under shade at room temperature for a period of seven to ten days. The dried plant was pulverized in a Willy mills. The pulverized plant (12 kg) was extracted three times with 80% methanol (30 liter) using maceration method at room temperature for 72 hours with occasional shaking. The combined filtrates were concentrated using rotary vacuum evaporator (Buchi, Switzerland) at 40-45 °C. This process resulted in the formation of 1.2 kg residue, which was the crude methanolic extract. 50 gm was separated from the crude extract and used for different biological activities. The remaining extract was dissolved in distilled water for fractionation into n-hexane, dichloromethane and ethyl acetate fractions on the basis of increasing polarity.
3.4 Fractionation process

The crude extract (1.2 kilogram) was macerated in one liter of distilled H₂O and shifted to a separating funnel for fractionation. About two liter of n-hexane was added to the separating funnel and agitated vigorously. After that agitation, the two layers were produced in the funnel when kept on stand. The upper layer was the n-hexane layer and was separated. This process was repeated three times, and the combined upper layers were concentrated using rotary evaporator at 40 °C to obtain about 22 grams of n-hexane fraction. After separation of hexane layer, the same procedure was followed for dichloromethane (DCM) and ethyl acetate (EtOAc) solvents which yielded about 65 grams of DCM fraction and 48 grams of EtOAc fraction and the remaining layer was considered as aqueous fraction. The whole scheme of fractionation is shown in Figure 2 (Kupchan and Tsou, 1973 and Wagenen et al., 1993). The crude extract and its three solvents fractions were used for various biological activities as well as isolation of phytochemical compounds.
Figure 3.2 Summary of Scheme used for fractionation process
5.3.5 Isolation and purification of compounds from fractions

DCM and EtOAc solvents fractions were showed significant results of *in-vitro* activities in the current study. Therefore, these fractions were selected for isolation of compounds. DCM fraction (50 gram) was subjected to column chromatography on silica gel using *n*-hexane with gradient of chloroform up to 100%. After running of column chromatography, six major sub-fractions (Fr.1-6) were collected according to TLC profiles. Sub-fraction 3 was loaded on silica gel (elution system: *n*-hexane: EtOAc at ratio of 8:2) to get compound 1. Sub-fraction 6 was also rechromatographed on silica gel and using *n*-hexane: EtOAc (7:3) to get compounds 2 and 3.

EtOAc fraction (30 gram) was rechromatographed on silica gel using *n*-hexane with gradient of EtOAc up to 100%. Matching the TLC results, five major sub-fractions (Fr.1-5) were collected. Sub-fraction 4 was rechromatographed on silica gel using *n*-hexane: CHCl₃ up to 100 %. This partition process provided (1-8) sub-fractions. Sub-fraction 3 was further loaded to c.c. on silica gel using *n*-hexane: EtOAc (3:7) to afford compound 4. Sub-fraction 5 was further loaded on sephadex LH- 20 and was eluted using hexane: EtOAc (2:8) solvent system to obtain compound 5. Sub-fractions 4, 6 and 7 were pooled together as they found similar spots on TLC. The combined sub-fractions were further rechromatographed over ODS RP-C₁₈ silica gel and using methanol: water (4:6) solvent system to get compound 6.
Figure 3.3 Isolation Scheme of Dichloromethane Fraction of *B. Procumbens*
Figure 3.4 Isolation Scheme Ethyl acetate Fraction of *B. Procumbens*
3.6 Chromatographic Techniques

Open column chromatography (OCC) was carried out on silica gel (63-200 μm: ODS RP-C\textsubscript{18}). Gel permeation chromatography (GPC) was performed on Sephadex LH-20 using various eluents. Thin layer chromatography (TLC) was performed using 60 F\textsubscript{254} and RP\textsubscript{18} silica gel. UV light (254 nm and 366 nm) was used for the visualization of TLC plates, sprayed with Ce (SO\textsubscript{4})\textsubscript{2} reagent for the detection of compounds (Nguyen, 2015).

3.7 Nuclear Magnetic Resonance (NMR)

The \textsuperscript{1}H and \textsuperscript{13}C-NMR spectra of the isolated compounds were operated on Bruker AV 300-600 (LC-NMR) and AMX 400,500 HD nuclear magnetic resonance spectrometers at frequency range between 300-600 MHz. The signals for primary (methine), secondary (methylene) and tertiary carbons (methyl) i.e. (CH, CH\textsubscript{2} and CH\textsubscript{3}) were recorded using a technique called Distortion-less enhancement by polarization transfer (DEPT). Quaternary carbons and all the multiplets of various carbons were visualized through decoupled \textsuperscript{13}C-NMR broad-band (BB) spectra. Tetra-Methyl-Silane (TMS) was used as internal standard. The \textsuperscript{1}H, \textsuperscript{13}C-NMR, ESI-MS and EI-MS spectra were recorded at HEJ Research Institute of Chemistry, University of Karachi, Pakistan.

3.8 Mass Spectrometry

For obtaining the electron ionization mass spectra (EI-MS) of the isolated compounds were recorded on a Finnigan MAT 312 spectrometer while electrospray ionization mass spectra (ESI-MS) were measured on QSTAR XL mass spectrometer.

3.9 Qualitative analysis of phytochemicals

3.9.1 Stock solution

The stock solution was prepared by methanolic crude extract of \textit{B. Procumbens}. The crude extract (1gram) was dissolved in 100 mL of the respective solvents.
The obtained stock solution was used for phytochemical screening (alkaloids, flavonoids, saponins, tannins, phenols, steroids and cardiac glycosides) by using the standard procedures.

3.9.2 Test for alkaloid

Crude extracts and solvents fraction (0.5 gram) of *B. Procumbens* was added with 8 ml of 1% HCl and heated the mixture, filtered and allowed to cool down. Mayer’s reagent and Dragendorff’s reagent were titrated against 2 ml of each of the filtrter samples. The cream or yellow precipitate indicated the presence of alkaloid compounds (Harborne, 1973).

3.9.3 Test for flavonoids

Sample plant (50 mg) was taken and 10 ml of distilled water were mixed in a test tube. Concentrated H$_2$SO$_4$ (5 ml) and dilute ammonia solution (5 ml) were added with 5 ml of each sample. The appearance of yellow color verified the presence of flavonoid (Sofowora, 1993).

3.9.4 Test for saponins

0.2 gram of sample plant were mixed with 20 ml of distilled water and boiled in a test tube on water bath. 5 ml of distilled water was mixed with 10 ml of the filtrate and and was shaken vigorously until the formation of stable persistent froth (Harborne, 1973).

3.9.5 Test for tannin

Each sample (50 mg) of *B. Procumbens* was dissolved in 20 ml of distilled H$_2$O. Then, added a few drops (0.1 % ferric chloride solution) to the filtrate of each sample. The Formation of blue black color indicated the presence of tannins (Sofowora, 1993).

3.9.6 Test for Phenol

500 mg samples of *B. Procumbens* were dissolved in 5 ml of dist.H$_2$O. The aqueous filerate of each sample was added with 2 ml of 5% ferric chloride solution in a test tube. The appearance of dark green colour indicated the presence of phenolic compounds (Sofowora, 1993).
3.9.7 Test for steroids

Add 2 ml of acetic anhydride and 2 ml conc. H\textsubscript{2}SO\textsubscript{4} to 5 ml of each sample. Change of colour from violet to blue confirms the presence of steroids (Boxi et al., 2010).

3.9.8 Test for cardiac glycosides

2 ml of glacial acetic acid containing 1 drop of ferric chloride solution and 1 ml of concentrated H\textsubscript{2}SO\textsubscript{4} were added to the extracts and fractions of studied plant in test tube. Brown ring indicates the presence of cardiac glycosides (Obianime and Uche, 2008).

3.10 Analysis of total phenolic content

The content of phenolics was determined in crude extracts and various fractions of \textit{B. procumbens} using folin ciocalteu reagent (Singleton et al., 1999). The methanolic solution of each samples (0.5 mL, 1 mg mL\textsuperscript{-1}) were added with folin reagent (2.5 ml, 10 \% diluted with distilled water) and then NaHCO\textsubscript{3} solution (2.5 ml, 7.5 \% NaHCO\textsubscript{3}) was added in a volumetric flask. After forty five minutes, the absorbance was measured using spectrophotometer at $\lambda_{\text{max}} = 765$ nm against a blank. The same method was followed for the standard solution of gallic acid, and the results were expressed in terms of gallic acid equivalent to mg of GA/g of samples. The samples were carried out in triplicate for each analysis.

3.11 Analysis of total flavonoid content

The content of flavonoids was determined in crude extracts and various fractions of studied plant according to Quettier et al., (2000). The methanol solution of each extract and fraction (1 ml, 1 mg ml\textsuperscript{-1}) was added with aluminium chloride solution (1 ml, 2 \% dissolved in methanol) in a volumetric flask. After an hour, the absorbance (Abs) was measured spectrophotometrically at 415 nm. The same procedure was followed for the standard solution of rutin, and the result was expressed in terms of rutin equivalent to mg of RU/g of samples. The analysis was carried out in triplicate for each sample.
3.12 Radical scavenging activity (DPPH)

The methanolic crude extracts and different solvent fractions of *B. Procumbens* was determined using DPPH radical scavenging assay (Jain *et al.*, 2008). The each samples was dissolved in methanol (3ml, 20-100 µg ml⁻¹) was added with DPPH solution (1ml, 0.1 mM). After 30 min, Abs was measured spectrophotometrically at 517 nm. The same procedure was followed for the standard of ascorbic acid. This activity was determined by the following formula such as:

\[
Radical\;scavenging\;activity\;(%)=\frac{A_0-A_1}{A_0}\times 100
\]

Where, \(A_0\) is the absorbance of control while \(A_1\) is the absorbance of samples or standard. IC₅₀ values were determined using a non-linear equation (% inhibition versus concentration).

3.13 Antimicrobial activity

3.13.1 Strains and culture media

The antimicrobial (Antibacterial and antifungal) assay of crude methanolic extract and different solvents fractions of *B. Procumbens* was tested against Gram-positive, Gram-negative bacteria and fungal strains. Bacterial strains were cultured and maintained on nutrient agar slants and incubated at 37 °C while fungal strains were cultured and maintained on potato dextrose agar at 28-30 °C.

3.13.2 Disc diffusion method

The antimicrobial activity (antibacterial and antifungal) was evaluated using standard protocol described by NCCLS, (2002). A sterile disc was impregnated with 20 µL of 100 mg of samples extract/ml and placed on the inoculated agar. Cefixime was used for bacteria as well as Clotrimazole was used for fungi. Followed by incubation of bacteria at 37 °C for 18-24 hrs whereas, the fungal strains were at 30°C for 24-72 hrs.
3.14 Brine shrimp lethality bioassay

Cytotoxicity activity was carried out for extract and fractions of sample plant using standard protocol (Atta-ur-Rahman et al., 2001 and Mayer et al., 1982). Brine shrimp (Artemia salina) eggs were stored at 4 °C. The hatching tray was filled with filtered brine solution. Eggs (50 mg) were sprinkled on the hatching try and left for incubation at 37°C. Stock solution was prepared by taking samples (20 mg) and dissolved in 2 ml of sterile DMSO. Stock solution (five μL, fifty μL and five hundred μL) were taken in three glass vials, at the concentration of ten, hundred and one thousand μg/mL. They were left overnight for solvent evaporation. Two days later of hatching process, 30 larvae were placed individually in the vials. Sea water was added to make the final volume of 5 mL. Loaded vials were incubated at 25-27 °C for 24 hrs. Extra two vials were taken, one containing the standard drug (Etoposide), and the other vial containing the respective solvent as negative control. Probit analysis was used to determine LD50 values (Kumarasamy et al., 2003).

3.15 Larvicidal activity

3.15.1 Stock solution preparation (1000 ppm)

250 mg of crude extract and corresponding fractions of studied plant were dissolved in 5 ml of ethanol and made up the volume with distilled water in 250 ml volumetric flask.

3.15.2 Procedure

The larvicidal activity of the sample plant was estimated against the larvae of Culex quinquefasciatus using standard method (WHO, 2005). 200, 300 and 400 μg/ml of different concentrations was prepared from the stock solution and was tested against the larvae of C. quinquefasciatus. 1 ml of ethanol was mixed with 249 ml water in a 300 ml beaker and this mixture was used as control. Twenty five 3rd instar larvae were introduced to each of the test solutions as well as control. Each concentration of the test solution as well as control was carried out in triplicate. The mortality of larvae was recorded after 24 hours of post treatment. The mortality data were subjected to probit
analysis (SPSS 1.5 Version software) to calculate lethal concentration values (LD$_{50}$) at 95% confidence limits.

3.16 Phytotoxicity Bioassay (*Lemna minor*)

Phytotoxic activity was performed to screen the various types of extract and fractions of *B. Procumbens* using *Lamna minor* plant (Atta-ur-Rahman, 1991). The required medium was prepared by mixing appropriate inorganic components in distilled water (100 ml) and pH (5.5-6.5) of medium was adjusted by adding KOH pellets. The medium was then sterilized by autoclaving for 15 min at 121°C. 20 mg/ml of samples were dissolved in methanol working as stock solution. Three sterilized flasks were used for each concentration and pipetted (ten μL, hundred μL and one thousand μL) into these flasks at the concentration of ten, hundred and one thousand μg/mL and allowed these flasks to evaporate methanol overnight. 20 ml of E-medium with the addition 3 healthy fronds (total fronds used 10) of the plant (*Lemma minor*) were added to each flask. These flasks were placed in the growth cabinet for 7 days by maintaining the ambient condition.

\[
% \text{ inhibition of growth} = 100 - \frac{\text{No. of fronds in tested sample}}{\text{No. of fronds in negative control}} \times 100
\]
3.17 Anti-Leishmanial activity

Isolated compounds of *B. Procumbens* (1, 2, 4, 5 & 6) were screened for leishmanicidal activity using standard protocol (Saeed et al., 2010; Khademvatan et al., 2011). Culture of promastigotes, *L. major* was incubated in RPMI-1640 at 22-25 °C. Heat-inactivated fetal bovine serum (10 %, 56°C for 30 min) was added into media. Culture was subjected to centrifuge (2000 rpm, 10 min) and treated with saline solution three times. The culture was then introduced with fresh culture medium to achieve a net density of 10⁶ cells/mL. Acquired medium (180 µl) was placed in the first row of a 96-well micro titer plate, and 100 µl of the same medium was placed in remaining rows. Test compounds (20 µl) was subjected into medium and diluted, serially. Afterwards, all the wells were supplemented with 100 ml of culture containing parasites. DMSO was used as control was placed in one row which received medium while one row was specified for standard drugs (Pantamidine).

3.18 *In-vitro* acetyl cholinesterase (AChE) inhibition assay

Isolated compounds (1, 4, 5 & 6) of *B. Procumbens* were assessed for their AChE inhibitory activities using Spectrophotometric method as described by Salles et al., (2003) & Ellman et al., (1961). Acetyl-thiocholine iodide (200 µl, 15 mM) was used for substrate. Reaction mixture containing 150 µl (100 mM) of sodium phosphate buffer (pH 8.0), Dithiobis-nitrobenzoic acid (DTNB) (10 µl, 3 mM), 10 µl (0.2 mM) of each test compounds solution and 20 µL of AChE solution, which were mixed and incubated (30 °C for 15 min). Furthermore, the mixture was examined spectrophotometrically (412 nm). Test compounds and the positive control (galanthamine) were dissolved in EtOH. Finally, the percentage AChE inhibitory concentration (% IA) was determined as following:

\[
% \text{IA} = \left[ \frac{(C_C - n \times C_e)}{C_C} \right] \times 100
\]

Where: \(C_C\) = control kinetic (all reactants, excluding AChE),

\(C_e\) = experimental kinetic (each sample concentration)
All experiments were carried out in triplicate. The IC$_{50}$ values for the tested compounds were determined by a linear regression analysis by using the Excel program (Microsoft Office).

### 3.20 Cytotoxic activity (NIH 3T3 cell lines)

Pure compounds of sample plant were evaluated against human B cell lymphocytes (15310-LN cell lines) for their cytotoxic activity using previously reported protocol (Afridi et al., 2015). Briefly, the test compounds (1-6) and reference molecule adamantane ethanol (AdEtOH) was evaluated against NIH-3T3 cell lines for their cytotoxicity potential using MTT assay in a 96-wells flat-bottom plate.

The cytotoxicity against human B cells expressing HLA-DRB1 class II antigen was determined. Flow cytometric technique based on propidium iodide (PI) staining was used. Shortly, 7×10$^4$ cells/well cultured cells were seeded in the presence of test samples and then incubated for 48 hours at 37 °C with 5% CO$_2$. After 48 hours incubation the cells were centrifuged at 1300 rpm for 5 min, washed with PBS and mixed with 5% fetal bovine serum (FBS) followed by PI (0.5 mg/mL) staining and flow cytometer (FACS caliber-BD) analysis. The cytotoxicity values were offered in percentage (dead cells) obtained from the histogram of propidium iodide-stained cell lines. Results were articulated as the percent growth inhibition and percent cell viability.

### 3.21 Treg cell proliferation assay

Treg cell assays were carried out as in the same way as described earlier (Afridi et al., 2015). Briefly, various concentrations of isolated compounds of *B. Procumbens* (1-6) were incubated with CD4+CD25+ markers expressing regulatory T cells for 24-48 h at a density of 7×10$^4$ cells/well (200 µL) in 96-well round-bottom plates. To estimate IL-2 release the culture supernatant (100 µL) was collected and proceeded using an IL-2 detection kit. The CD4+CD25+ T cell proliferation induced by the isolated compounds of *B. Procumbens* was determined by measuring the intensity of BrdU dye incorporation using a BrdU cell proliferation kit as described previously (Hori et al., 2003). T cell proliferation was expressed as percentage values.
3.22  *In-vivo* pharmacological methods

3.22.1 Animals used

Wistar rats and mice “BALB/c” were used for *in-vivo* activity. Animals were housed at ambient conditions and 12 hrs day and night cycle for one week. They were given free access to a standard laboratory feed, water and ad-libitum to acclimatize to the environmental conditions. Guidelines of the Commission of Life Sciences, National Research Council and Institute of Laboratory Animal Resources (IOLAR) were strictly followed during the experiments (Khan *et al.*, 2012). The experimental protocols for *in-vivo* experiments were approved by the bioethical committee of the Department of Centre of Biotechnology and Microbiology University of Peshawar, Pakistan (registration number: 215/COBAM/Proj).

3.22.2 Acute toxicity

*In-vivo* acute toxicity was carried out for crude extract of *B. procumbens* using standard protocols (Araujo *et al.*, 2014). 500, 1000, and 2000 mg/kg of various concentration of crude extract were given orally to BALB/c mice of either sex. Normal (10 ml/kg) saline received control group. The animals were observed carefully during 24 hours for any sign of toxicity.

3.22.3 Analgesic activity (anti-nociceptive)

Crude extract of sample plant was checked for their antinociceptive activity using the following procedures models.

3.22.3.1 Acetic acid induced writhing

The anti-nociceptive activity of *B. Procumbens* extract was performed using acetic acid induced abdominal constriction in mice (Koster *et al.*, 1959; Adzu *et al.*, 2001). The BALB/c mice were selected for this experiment and divided into 5 groups containing each group of six animals. Normal saline (10 ml/kg, *i.p.*) was given to group I. There are three different doses (100, 200, and 400 mg/kg, *i.p.*) of crude extract was received to groups II- IV, respectively. The standard drug (diclofenac Na, 10 mg/kg) was given by intraperitonially (*i.p.*) route to group V. All animals were induced by intraperitonal
injection of 1% acetic acid after thirty minutes of treatment. The writhing (muscular contractions) number was counted after 5 min of acetic acid injection and was noted up to 20 min.

\[
\% \text{ Analgesic effect} = 100 - \frac{\text{No. of writhings in tested animals}}{\text{No. of writhings in control animal}} \times 100
\]

3.22.3.2 Thermal nociception (hot-plate test)

*B. procumbens* crude extract was tested for central analgesic effect using hot plate model (Dar et al., 2005). The pre-screened animals was divided into 5 groups (n= 6). Test groups (II-IV), control (normal saline, group-I) received their respective doses as mentioned earlier, and the group V received Tramadol at dose of 20 mg/kg s.c. The animals were placed on hot metal plate (50 ± 0.05 °C), after thirty minute of treatment. The central analgesic of mice was observed in this experiments includes jumping, licking or flicking of hind limb. Reaction time for each group was recorded at 0, 30, 60, 90 and 120 min during the observation period.

\[
\% \text{ Analgesic effect} = \frac{\text{latency time of test} - \text{latency time of control}}{\text{cut off time} - \text{latency time of control}} \times 100
\]

3.23 Anti-inflammatory activity

BALB/c mice were used for investigation of *B. procumbens* extract for their possible anti-inflammatory effect using standard method (Khan et al., 2009). In this experiment, 5 groups of animals were used, containing six animals in each group. Test groups (II-IV), control (normal saline, group-I) received their respective doses, and group V received Indomethacin (10 mg/kg s.c). After 30 minutes of treatment, carrageenan (0.05 ml of 1 % solution) was injected subcutaneously to the right hind paw of each mouse. Digital plethysmometer using to measure the paw volume at 1- 5 hours. % inhibition of edema was determined through comparison of the average paw swelling in sample treated and standard treated groups with that of control.

\[
\text{Percent inhibition} = \frac{\text{Edema volume of control} - \text{Paw edema of tested gp}}{\text{Edema volume of control}} \times 100
\]
3.24  Hepatoprotective activity

3.24.1 Animals used

Wistar rats (150-200 g) were used and were housed at ambient conditions for one week. They were allowed free access to a standard laboratory feed, water and *ad-libitum* to acclimatize to the environmental conditions.

Experimental design

3.24.3 Carbon tetrachloride (CCl\textsubscript{4}) induced hepatotoxicity

Strain of wistar rats (150-200 g) were selected and divided in to 5 groups, each group containing 6 animals. Gum acacia reagent (5 %) was used as a vehicle control for suspending the standard drugs and the isolated compound (eupalitin-3-O-β-D-galactcopyranoside) was used in a low and high dose in the present study (De *et al.*, 2017).

**Group I** Control (vehicle): Received a single daily dose of 5 % suspension of gum acacia (1 ml/kg body weight, orally) for 5 consecutive days.

**Group II** (CCl\textsubscript{4}): Received CCl\textsubscript{4} (2.5 mg/kg body weight, orally) at single daily dose for 5 consecutive days.

**Group III** (standard): Received Silymarin (25 mg/kg body weight, orally) at single daily dose for 5 consecutive days.

**Group IV** (Tested compound): Received isolated compound of eupalitin-3-O-β-D-galactcopyranoside (30 mg/kg b.w, orally) at single daily dose for 5 consecutive days.

**Group V** (Tested compound): Received isolated compound i.e. eupalitin-3-O-β-D-galactcopyranoside (60 mg/kg b.w, orally) at single daily dose for 5 consecutive days.

Group I received liquid paraffin (1 ml/kg, subcutaneous) on the 2\textsuperscript{nd} and 3\textsuperscript{rd} day whereas; group II to group V received a single dose of CCl\textsubscript{4} with liquid paraffin (1: 1 ratio) on the 2\textsuperscript{nd} and 3\textsuperscript{rd} day.
3.24.4 Histology

Rats were scarified after 24 hrs following the last treatment. After this, Blood collection was accomplished and after clotting, serum was separated by centrifugation at 3000 rpm for 20 minutes and subjected to bio-chemical investigations. In addition, livers of the animals were removed and washed with 0.9 % saline. Then, 2-3 pieces of about 6 mm³ sizes were sliced and subsequently fixed in 10 % phosphate-buffered formaldehyde solution. After embedding in paraffin wax, thin sections of about 5 μm thickness were cut and stained with hematoxylin and eosin dye (Subash, et al., 2011).

3.24.5 Biochemical investigations

Bio-chemical parameters including serum enzyme such as ALP (Bergemeyer, 1974), SGPT, SGOT (Henry and Cannon, 1974) and total bilirubin (Mallory and Evenlyn, 1937) were determined using various assay kits supplied by the manufacturer. The biochemical analysis was performed using double beam UV/Visible spectrophotometer.

3.25 Proximate Composition

*B. Procumbens* plant and its roots were used for proximate composition to determine moisture, inorganic matter, ether extract oil, crude protein, dietary fiber and nitrogen-free extract using methods described by AOAC, (2005).

3.25.1 Moisture contents

2.0 gram of ground sample plant and its roots was taken into a silica dish previously dried and weighed. The required samples were then dried in an oven (100˚C for 3 hours). After drying, the samples were removed from the oven, cooled in a desiccator and weighed. The drying and weighing continued until a constant weight was obtained.

\[
Percent \, Moisture = \frac{Sample + Wt \, dish \, before \, drying - sample \, after \, drying}{Weight \, of \, sample} \times 100
\]

3.25.2 Inorganic matter

Crucible dishes were cleaned and dried in an oven. *B. procumbens* plant and its roots (2.0 g) were weighed into a pre-heated crucible. Ignite the samples in the dishes over a
burner flame, until it is charred completely. The whitish-grey residues were obtained, when the dishes were placed into a muffle furnace (560˚C for 2 hrs).

\[
\text{Percent Ash} = \frac{\text{Wt of dish} + \text{content after drying} - \text{wt of empty dish}}{\text{Wt of sample}} \times 100
\]

### 3.25.3 Ether extract

Samples were taken (2-4 gram) in extraction thimble using Soxhlet’s apparatus. The thimble was placed in extraction unit and connected it with round bottom flask (250 ml) using petroleum ether for extraction. Siphoning would occur after 5-6 min, when the extraction continued for 5-6 hrs. The flask was dried (105 ˚C), cooled and weighed again after completion of the process.

\[
\text{Ether extract} = \frac{\text{Wt of flask} + \text{fat} - \text{wt of empty flask}}{\text{Wt of sample}} \times 100
\]

### 3.25.4 Dietary Fiber

Fat-free samples (2.0 g) were weighed into a conical flask/beaker. Then, added sulphuric acid solution (200 ml, 1.25%) and boiled gently for 30 mins, maintaining constant volume of acid by the addition of hot water. The flask was removed, filtered and washed with boiling water until no longer acidic to litmus paper. Then, added sodium hydroxide solution (200 ml, 1.25%) and boiled for another 30 mins. After completion of boiling, the cooled samples were again filtered immediately. The insoluble residue was then transferred to the sintered crucible, washed three times (diethyether) and then dried in an oven at 150˚C unti to a constant weight. The crucible was placed in muffle furnace for incineration (560˚C, 1 hrs).

\[
\text{Crude fiber (％)} = \frac{\text{Wt of residue before ashing} - \text{wt of residue after ashing}}{\text{Weight of sample}} \times 100
\]

### 3.25.5 Crude protein

Crude protein was determined according to Kjeldahl method including digestion system and distillation unit with Titration. % Nitrogen of samples was multiplied by 6.25 factor to calculate the Crude Protein.
Where N: Normality of the acid, V: volume of the digest after dilution, D: sample dilution and % crude protein = % N x 6.25

3.25.6 Nitrogen-free extract

The Nitrogen-free extract can be found by difference. This value is obtained by substracting the sum of the percentages of moisture content, inorganic matter, ether extract, crude protein, and dietary fiber as described by James, (1995).

Nitrogen-free extract (carbohydrates) = 100 – (% moisture + % crude protein + % ether extract + % inorganic matter + % crude fiber).

3.26 Minerals Composition

3.26.1 Dry Ashing

The ash solution was prepared (AOAC, 2000) with slight modifications (Hussain et al., 2005). 2 gram of sample plant and its roots was taken into a crucible and ignite the samples over a burner flame, until it is charred completely. After charring, the carbon-free samples were shifted into muffle furnace (600ºC, 5 hrs) until it tured to grey-white residue. This residue was dissolved in 2 mL concentrated nitric acid and heated on a low flame for 1 minute. The dissolved residue was cooled, filtered and volume was made up with deionized water in volumetric flask (50 ml). Similar experimental treatment was followed for blank sample. The analysis of samples was carried out in triplicates.

3.26.2 Instrumentation

Iron, copper, zinc, chromium, nickel, cadmium and lead concentrations were determined by Atomic Absorption Spectrometer while concentrations of macro-minerals (sodium, potassium and calcium) using Flame Photometer as instrumental operations are summarized in Table 3.1.
Table 3.1 Instrumental condition for working of micro- and macro-minerals

<table>
<thead>
<tr>
<th>Micro-minerals</th>
<th>Lamp current (mA)</th>
<th>Wavelength (nm)</th>
<th>Silt width (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cd</td>
<td>7.5</td>
<td>228.8</td>
<td>1.3</td>
</tr>
<tr>
<td>Cr</td>
<td>7.5</td>
<td>359.3</td>
<td>1.3</td>
</tr>
<tr>
<td>Cu</td>
<td>7.5</td>
<td>324.8</td>
<td>1.3</td>
</tr>
<tr>
<td>Fe</td>
<td>10</td>
<td>248.3</td>
<td>0.2</td>
</tr>
<tr>
<td>Ni</td>
<td>10</td>
<td>232</td>
<td>0.2</td>
</tr>
<tr>
<td>Pb</td>
<td>7.5</td>
<td>283.3</td>
<td>1.3</td>
</tr>
<tr>
<td>Zn</td>
<td>10</td>
<td>213.8</td>
<td>1.3</td>
</tr>
<tr>
<td>Macro-minerals</td>
<td>Filter used</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ca</td>
<td>Ca filter</td>
<td>422.7</td>
<td>0.7</td>
</tr>
<tr>
<td>K</td>
<td>K filter</td>
<td>766.5</td>
<td>2.0</td>
</tr>
<tr>
<td>Na</td>
<td>Na filter</td>
<td>589</td>
<td>0.2</td>
</tr>
</tbody>
</table>

3.27 Amino Acid Composition

3.27.1 Sample Preparation

The reported method in HPLC Amino Acid Analysis system application data book (Shimadzu) used for normal hydrolysis. 23.30 mg of studied plant and its roots were taken in separate digestion tube. Then, added 2 ml of 6 N HCl and placed for 18-24 hours at 110 ºC under vacuum. The water was used to wash the hydrolyzed samples and evaporated on a rotary evaporator. Final volume was made up with 10 ml of deionized water. Before the injection of samples (20 µL) into the amino acid analyzer, it is filter and diluted with buffer A solution in a sample vial.

3.27.2 Instrumental Conditions

Amino acids composition was carried out on Amino Acid Analyzer (Shimadzu) with Shim-Pack Amino-Na column (4.6 mm, I.D x100 mm). Auto injector was used for the injected samples of *B. procumbens* and its roots. A gradient program of 72 minutes was set for mobile phase A, B, C with the initial flow rate of 0.4 ml/min. Ammonia trap column was used prior to column elution.
4. RESULTS

4.1 Chemical compositions

4.1.1 Proximate composition

Proximate analysis including moisture, inorganic matter (ash), crude protein, ether extract oil, crude fiber and nitrogen-free extract (NFE) of aerial parts and roots of *B. procumbens* is presented in Table 4.1. The moisture value of the aerial parts (9.29 ± 0.05 %) was found higher than the roots (7.36 ± 0.04%). The amount of protein and inorganic matter of aerial parts were reported significantly lower as compared to roots. The NFE and ether extract oil contents were not found significantly different (p>0.05) in sample plant. 4.35 ± 0.03% of crude fiber of aerial parts was recorded higher than the roots.

<table>
<thead>
<tr>
<th>Compositions</th>
<th>Aerial parts</th>
<th>Roots</th>
</tr>
</thead>
<tbody>
<tr>
<td>Moisture</td>
<td>9.29 ± 0.05\textsuperscript{a}</td>
<td>7.36 ± 0.04\textsuperscript{b}</td>
</tr>
<tr>
<td>Inorganic matter</td>
<td>2.18 ± 0.04\textsuperscript{b}</td>
<td>21.25 ± 0.03\textsuperscript{a}</td>
</tr>
<tr>
<td>Crude protein</td>
<td>6.78 ± 0.05\textsuperscript{b}</td>
<td>37.46 ± 0.02\textsuperscript{a}</td>
</tr>
<tr>
<td>Crude fiber</td>
<td>4.35 ± 0.03\textsuperscript{a}</td>
<td>0.96 ± 0.02\textsuperscript{b}</td>
</tr>
<tr>
<td>Crude fat</td>
<td>1.84 ± 0.05\textsuperscript{a}</td>
<td>1.75 ± 0.03\textsuperscript{a}</td>
</tr>
<tr>
<td>Nitrogen-free extract</td>
<td>75.56 ± 0.046\textsuperscript{b}</td>
<td>31.22 ± 0.05\textsuperscript{a}</td>
</tr>
</tbody>
</table>

Values are mean ± SD; n = 3. Means followed by different superscript in the same row are significantly different (p<0.05).

4.1.2 Mineral composition

The mineral composition of Aerial parts and roots of *B. Procumbens* is presented in Figure 1. The reported value of sodium was not found significantly different (p>0.05) in the sample plant of aerial parts and roots. The calcium (309.73 ± 0.06 mg/100g) and potassium (K) contents (274.59 ± 0.08 mg/100g) of aerial parts were significantly higher (p<0.05) as compared to roots (102.27 ± 0.02 mg/100g & 92.080 ± 0.04 mg/100g). The iron value of aerial parts (32.577 ± 0.05 mg/100g) was found lower than the roots (80.247 ± 0.04 mg/100g). The concentration of lead metal was found within the permissible limits, where as cadmium metal was not detected in the aerial parts as well as roots of *B. Procumbens.*
Figure 4.1 Mineral composition of aerial parts and roots of *B. procumbens* (Bar shows LSD value at P<0.05)

### 4.1.3 Amino acid composition

Amino acid composition of aerial parts and roots of *B. Procumbens* is presented in Table 4.2. Concentration of arginine (8.89 ± 0.08 g/100g) was found higher in roots as compared to aerial parts (3.49 ± 0.08 g/100g). Aerial parts and roots of studied plant were not found significantly different (p>0.05) in the composition of histidine. The values of phenylalanine and tryptophan were found higher in roots as compared to aerial parts of *B. Procumbens*. Comparatively higher concentration of aspartic acid, glutamic acid and alanine was found in aerial parts as compared to the roots.
Table 4.2  Amino acid composition (g/100 g) of the aerial parts and roots of *B. procumbens* | | Aerial parts | Roots |
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Essential amino acid</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Arginine</td>
<td>$3.49 \pm 0.08^b$</td>
<td>$8.89 \pm 0.08^a$</td>
</tr>
<tr>
<td>Histidine</td>
<td>$0.65 \pm 0.07^a$</td>
<td>$0.65 \pm 0.07^a$</td>
</tr>
<tr>
<td>Leucine</td>
<td>$0.75 \pm 0.08^a$</td>
<td>$0.66 \pm 0.08^a$</td>
</tr>
<tr>
<td>Lysine</td>
<td>$0.29 \pm 0.06^b$</td>
<td>$4.77 \pm 0.09^a$</td>
</tr>
<tr>
<td>Methionine</td>
<td>$1.23 \pm 0.04^b$</td>
<td>$3.73 \pm 0.09^a$</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>$1.77 \pm 0.08^b$</td>
<td>$3.36 \pm 0.10^a$</td>
</tr>
<tr>
<td>Threonine</td>
<td>$0.48 \pm 0.06^b$</td>
<td>$3.52 \pm 0.07^a$</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>$6.23 \pm 0.06^b$</td>
<td>$8.82 \pm 0.07^a$</td>
</tr>
<tr>
<td>Valine</td>
<td>$3.60 \pm 0.08^a$</td>
<td>$2.83 \pm 0.09^b$</td>
</tr>
<tr>
<td><strong>Non-essential amino acid</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aspartic acid</td>
<td>$31.25 \pm 0.08^a$</td>
<td>$26.18 \pm 0.07^b$</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>$25.27 \pm 0.06^a$</td>
<td>$17.66 \pm 0.08^b$</td>
</tr>
<tr>
<td>Alanine</td>
<td>$12.74 \pm 0.12^a$</td>
<td>$7.83 \pm 0.10^b$</td>
</tr>
<tr>
<td>Glycine</td>
<td>$7.69 \pm 0.06^a$</td>
<td>$5.65 \pm 0.07^b$</td>
</tr>
<tr>
<td>Proline</td>
<td>$0.00 \pm 0.00^b$</td>
<td>$0.01 \pm 0.06^a$</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>$0.81 \pm 0.06^a$</td>
<td>$0.48 \pm 0.06^b$</td>
</tr>
<tr>
<td>Cysteine</td>
<td>$0.31 \pm 0.07^b$</td>
<td>$1.78 \pm 0.05^a$</td>
</tr>
</tbody>
</table>

Values are mean ± SD; *n* = 3. Means followed by different superscript in the same row are significantly different (p<0.05).

**Figure 4.2** Chromatogram of Amino acid profile
4.1.4 Qualitative analysis of phytochemicals

Various phytochemical compounds were screened qualitatively in crude extract and different fraction of *B. procumbens* (Table 4.3). The phenols, flavonoids and cardiac glycoside compounds were present in crude extract of *B. Procumbens*. Flavonoid and glycoside constituents were detected in ethyl acetate fraction. The flavonoids, phenols, alkaloids, tannins and steroids compounds were not detected in n-hexane fraction.

<table>
<thead>
<tr>
<th>Phytochemical compounds</th>
<th>Test names</th>
<th>CME</th>
<th>HxF</th>
<th>DCMF</th>
<th>EtOAcF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alkaloids</td>
<td>Mayer’s test</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Dragendorff’s test</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>Phenols</td>
<td>Ferric chloride test</td>
<td>+++</td>
<td>−</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>Flavonoids</td>
<td>Ammonia test</td>
<td>+++</td>
<td>−</td>
<td>++</td>
<td>+++</td>
</tr>
<tr>
<td>Cardiac Glycoside</td>
<td>Ferric chloride test</td>
<td>+++</td>
<td>+</td>
<td>++</td>
<td>+++</td>
</tr>
<tr>
<td>Tannins</td>
<td>Ferric chloride test</td>
<td>++</td>
<td>−</td>
<td>−</td>
<td>++</td>
</tr>
<tr>
<td>Steroids</td>
<td>Liebermann-Burchard test</td>
<td>+</td>
<td>−</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>Saponins</td>
<td>Frothing test</td>
<td>+</td>
<td>+++</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>


(++)+: Very High, (++): High (+): Moderate and (−): Absent

4.1.5 Quantitative analysis of phytochemicals

The total phenols and flavonoid compounds in crude extracts and different solvent fractions of *B. Procumbens* are presented in table 4.4 and figure 4.3. The phenolic compounds among the various extracts and fractions were expressed in terms of gallic acid (GA) using standard curve equation, \( \text{Abs} = 0.0065[\text{GA}] + 0.0181, R^2 = 0.9915 \). The phenolic content was reported higher in crude extract (82.80 mg GA/g) and ethyl acetate fraction (118.45 mg GA /g) followed by DCM fraction (68.82 GA /g) of *B. Procumbens*. This data are summarized in Table 4.6.
Table 4.4  
**Absorbance of standard compound (Gallic acids)**

<table>
<thead>
<tr>
<th>Gallic acids concentration (mg/ml)</th>
<th>Absorbance (mean value) at $\lambda_{\text{max}} = 765\text{nm}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>20</td>
<td>0.17</td>
</tr>
<tr>
<td>40</td>
<td>0.26</td>
</tr>
<tr>
<td>60</td>
<td>0.44</td>
</tr>
<tr>
<td>80</td>
<td>0.52</td>
</tr>
<tr>
<td>100</td>
<td>0.66</td>
</tr>
</tbody>
</table>

Figure 4.3  
Calibration curve of Gallic Acids

The flavonoids content of extracts and different fractions were expressed in terms of quercetin (QE) using standard curve equation, $\text{Abs} = 0.0042[\text{QE}] + 0.0138$, $R^2 = 0.9829$ (table 4.5 & Figure 4.4). The flavonoid content was also found higher in crude extract (27.72 mg QE/g) and ethyl acetate fraction (34.42 mg QE/g) of *B. Procumbens* (Table 4.6).

Table 4.5  
**Absorbance of standard compound (Quercetin)**

<table>
<thead>
<tr>
<th>concentration (mg/ml)</th>
<th>Absorbance (mean value) at $\lambda_{\text{max}} = 415\text{nm}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>20</td>
<td>0.12</td>
</tr>
<tr>
<td>40</td>
<td>0.19</td>
</tr>
<tr>
<td>60</td>
<td>0.25</td>
</tr>
<tr>
<td>80</td>
<td>0.32</td>
</tr>
<tr>
<td>100</td>
<td>0.45</td>
</tr>
</tbody>
</table>
Table 4.6  Total phenolic and flavonoids compounds of crude extracts and fractions of *B. Procumbens*

<table>
<thead>
<tr>
<th>Extracts &amp; Fractions</th>
<th>Total Phenolic (mg of Gallic acid/g of sample)</th>
<th>Total Flavonoid (mg of Quercetin /g of sample)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methanolic extract</td>
<td>82.80 ± 0.08</td>
<td>27.72 ± 0.06</td>
</tr>
<tr>
<td>Hexane fraction</td>
<td>8.23 ± 0.08</td>
<td>14.42 ± 0.41</td>
</tr>
<tr>
<td>Dichloromethane fraction</td>
<td>68.82 ± 0.07</td>
<td>18.55 ± 0.07</td>
</tr>
<tr>
<td>Ethyl acetate fractions</td>
<td>118.45 ± 0.07</td>
<td>34.42 ± 0.06</td>
</tr>
<tr>
<td>Leaves extract</td>
<td>20.48 ± 0.06</td>
<td>15.28 ± 0.07</td>
</tr>
<tr>
<td>Roots extract</td>
<td>17.23 ± 0.07</td>
<td>8.35 ± 0.07</td>
</tr>
</tbody>
</table>

All values are expressed as mean ± standard deviation (n = 3).

4.2  *In-vitro* Biological activities

4.2.1  Radical scavenging activity

Samples were tested in different concentrations for antioxidant activity (DPPH model) (Table 4.7). The EtOAc fraction showed maximum DPPH scavenging activity (82.22 %) followed by hexane fraction (64.78%). The crude extract of the sample plant possessed moderate antioxidant effect (55.44%). The IC$_{50}$ values for ethyl acetate, hexane, and DCM were 40.24, 73.88 and 87.68 µg/mL, respectively, while the methanol extract had an IC$_{50}$ of 120 µg/ml as compared to standard ascorbic acid (28.78 µg/ml)
Table 4.7  Radical scavenging activity of crude extracts and fractions of *B. procumbens*

<table>
<thead>
<tr>
<th>Solution</th>
<th>% Scavenging activity</th>
<th>Standard</th>
</tr>
</thead>
<tbody>
<tr>
<td>Conc (μg/mL)</td>
<td>Methanol extract</td>
<td>Hexane fraction</td>
</tr>
<tr>
<td>20</td>
<td>4.12 ± 0.42</td>
<td>18.12 ± 0.84</td>
</tr>
<tr>
<td>40</td>
<td>16.22 ± 0.52</td>
<td>24.23 ± 0.45</td>
</tr>
<tr>
<td>60</td>
<td>27.45 ± 0.56</td>
<td>46.31 ± 0.53</td>
</tr>
<tr>
<td>80</td>
<td>42.33 ± 0.63</td>
<td>53.74 ± 0.64</td>
</tr>
<tr>
<td>100</td>
<td>55.44 ± 0.65</td>
<td>64.78 ± 0.76</td>
</tr>
<tr>
<td>Control</td>
<td>1.02</td>
<td>0.11</td>
</tr>
<tr>
<td>IC&lt;sub&gt;50&lt;/sub&gt; (μg/mL)</td>
<td>120.27</td>
<td>73.88</td>
</tr>
</tbody>
</table>

4.2.2  Antimicrobial activity

4.2.2.1  Anti-bacterial activity

Crude extract and solvent fractions were screened for antibacterial activity (Table 8). The DCM and EtOAc fractions (12 ± 0.12 mm and 10.6 ± 0.01 mm, respectively) showed good zone of inhibition followed by hexane fraction (9.5 ± 0.05 mm) against *X. campestris*. The ethyl acetate and hexane fractions exhibited maximum activity against *E.coli*. The DCM fraction significantly inhibited the growth of *C. michiganensis* having zone of inhibition of 12.2 ± 0.21 mm. However, the crude extract, hexane, DCM & EtOAc fractions exhibited weak activity against *K. pneumonia*.

Table 4.8  Zone of inhibitory activity of crude extract and subsequent fractions of *B. Procumbens*

<table>
<thead>
<tr>
<th>Bacterial strains</th>
<th>Crude extract (mm)</th>
<th>Hexane fract (mm)</th>
<th>DCM fract (mm)</th>
<th>EtOAc fract (mm)</th>
<th>*Standard (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>X. campestris</em></td>
<td>3.5 ± 0.04</td>
<td>9.5 ± 0.05</td>
<td>12 ± 0.12</td>
<td>10.6 ± 0.01</td>
<td>13.4 ± 0.07</td>
</tr>
<tr>
<td><em>P. vulgaris</em></td>
<td>0 ± 0.00</td>
<td>7.5 ± 0.03</td>
<td>7.2 ± 0.13</td>
<td>6.4 ± 0.14</td>
<td>11.3 ± 0.12</td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td>5 ± 0.14</td>
<td>11.2 ± 0.07</td>
<td>8.4 ± 0.15</td>
<td>11.8 ± 0.14</td>
<td>12.2 ± 0.14</td>
</tr>
<tr>
<td><em>S. aureus</em></td>
<td>9.0 ± 0.26</td>
<td>8.6 ± 0.26</td>
<td>10.2 ± 0.26</td>
<td>8.1 ± 0.21</td>
<td>11.4 ± 0.20</td>
</tr>
<tr>
<td><em>K. pneumonia</em></td>
<td>3.8 ± 0.10</td>
<td>2.8 ± 0.10</td>
<td>6.4 ± 0.30</td>
<td>8.5 ± 0.20</td>
<td>20.3 ± 0.10</td>
</tr>
<tr>
<td><em>P. aerugonosa</em></td>
<td>10.5 ± 0.10</td>
<td>12.5 ± 0.30</td>
<td>7.2 ± 0.10</td>
<td>12.4 ± 0.16</td>
<td>20.3 ± 0.10</td>
</tr>
<tr>
<td><em>B. subtilis</em></td>
<td>9.3 ± 0.16</td>
<td>15.6 ± 0.16</td>
<td>14.3 ± 0.20</td>
<td>15.6 ± 0.21</td>
<td>21.3 ± 0.16</td>
</tr>
<tr>
<td><em>C. michiganensis</em></td>
<td>7.0 ± 0.14</td>
<td>6.5 ± 0.07</td>
<td>12.2 ± 0.21</td>
<td>8.5 ± 0.07</td>
<td>12.6 ± 0.10</td>
</tr>
</tbody>
</table>

*Standard: Cefixime*
Zone of inhibition of DCM & EtOAc fractions against *X. campestris*  
Zone of inhibition of EtOAc, Hexane and DCM fractions against *E. coli*

**Figure 4.5** Antibacterial activity of various fractions of *B. Procumbens* against *X. campestris* and *E. coli*

### 4.2.2.2 Anti-fungal activity

*B. procumbens* extracts and various fractions were screened for anti-fungal activity and showed varied level of growth inhibition of strains as presented in Table 4.9. The hexane fraction showed nearly similar inhibition of *F. oxysporum* (23.2 ± 0.20 mm) when compared to standard (23.6 ± 0.16 mm). EtOAc fraction also displayed considerable inhibition of growth (13.7 ± 0.16 mm) against *F. solani*. The crude extract, DCM and EtOAc fractions showed no activity against *A. niger* and *A. flavus*

**Table 4.9**   **Antifungal activity of crude extract and different fractions of *B. Procumbens***

<table>
<thead>
<tr>
<th>Fungal strains</th>
<th>Crude extract (mm)</th>
<th>Hexane frac (mm)</th>
<th>DCM frac (mm)</th>
<th>EtOAc frac (mm)</th>
<th>*Standard (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>F. oxysporum</em></td>
<td>14.8 ± 0.36</td>
<td>23.2 ± 0.26</td>
<td>12.5 ± 0.31</td>
<td>20.5 ± 0.31</td>
<td>23.6 ± 0.16</td>
</tr>
<tr>
<td><em>F. solani</em></td>
<td>8.6 ± 0.21</td>
<td>10.4 ± 0.20</td>
<td>7.7 ± 0.16</td>
<td>13.7 ± 0.16</td>
<td>15.4 ± 0.21</td>
</tr>
<tr>
<td><em>A. niger</em></td>
<td>0.0 ± 0.00</td>
<td>9.5 ± 0.26</td>
<td>0.0 ± 0.00</td>
<td>0.0 ± 0.00</td>
<td>17.4 ± 0.30</td>
</tr>
<tr>
<td><em>M. piriformis</em></td>
<td>8.6 ± 0.26</td>
<td>11.4 ± 0.10</td>
<td>0.0 ± 0.00</td>
<td>9.7 ± 0.10</td>
<td>24.5 ± 0.20</td>
</tr>
<tr>
<td><em>A. flavus</em></td>
<td>0.0 ± 0.00</td>
<td>9.5 ± 0.31</td>
<td>0.0 ± 0.00</td>
<td>0.0 ± 0.00</td>
<td>15.5 ± 0.15</td>
</tr>
</tbody>
</table>

*Standard: Clotrimazole*
4.2.3 Cytotoxic activity (Brine shrimp)

The cytotoxic bioassay (brine shrimp lethality assay) was carried out using crude extracts and fractions of *B. Procumbens*. Various concentrations (10, 100, and 1000 µg/mL) of the all samples were used for their cytotoxic effect and etoposide was used as a standard drug (Table 4.10). Roots extract showed good cytotoxicity (73.33%) at the highest test concentration (1000 µg/mL) while mild activity (40 and 30%) was observed at both lower concentrations (100 and 10 µg/mL) respectively. The DCM fraction exhibited significant activity (90%) at high concentration (1000 µg/mL) whereas, moderate cytotoxic effect (60 and 10%) was observed at lower concentrations (100 and 10 µg/mL) respectively. Ethyl acetate fraction showed an appreciable activity (57%) at the highest test concentration (1000 µg/mL) while, having moderate potential (43 and 36%) at lower concentrations (100 and 10 µg/mL), respectively. The crude extract of *B. Procumbens*, leaves extract and hexane fraction showed no cytotoxicity at 1000, 100 and 10 µg/mL in this study. A maximum cytotoxic action was observed for roots extract, DCM fraction, and ethyl acetate fraction with LD$_{50}$ values 148.12, 175.04, and 279.95 µg/mL, respectively.

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Dose (µg/ml)</th>
<th>No. of shrimps</th>
<th>No. of survived shrimps</th>
<th>No. of dead shrimps</th>
<th>LD$_{50}$ (µg/ml)</th>
<th>Std. Drug</th>
<th>LD$_{50}$ (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>C. extract of sample plant</td>
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</tr>
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<td>30</td>
<td>0</td>
<td>0</td>
<td>0.00</td>
<td>Etoposide</td>
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<td>0.00</td>
<td>Etoposide</td>
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<td>DCM fraction</td>
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<td>1000</td>
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<td>0.00</td>
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<td>9</td>
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</tbody>
</table>
4.2.4 Larvicidal Activity

Sample plant extract and its different solvent fractions were examined for their larvicidal effect against the 3rd instar larvae of *Culex quinquefasciatus* after 24 hrs of exposure. The larvicidal activity of all the samples was conducted at various concentrations (200, 300 and 400 μg/ml). The larvicidal effect of all samples in the experiment was compared with positive control (ethanol) (Table 4.11). The leaf extract and hexane fraction showed highest percent mortality (100% and 96%) at high concentration (400 μg /ml). The DCM fractions and crude extract also displayed maximum percent mortality (76 and 68%) at high concentration of 400 μg /ml whereas, EtOAc fraction showed moderate mortality (52%) at the same concentration. The LD$_{50}$ values for leaf extract, hexane fraction, DCM fraction and crude extract against the larvae of *C. quinquefasciatus* were 26.84, 46.98, 223.43 and 326.35 μg /ml.

**Table 4.11 Larvicidal activity of crude extracts and fractions of *B. Procumbens***

<table>
<thead>
<tr>
<th>Samples</th>
<th>Conc (μg/ml)</th>
<th>No. of larvae</th>
<th>Dead larvae after 24 Hr</th>
<th>% Mortality</th>
<th>LD$_{50}$ (μg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude extract of BP</td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>200</td>
<td>25</td>
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</tr>
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<td>400</td>
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<tr>
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</tr>
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<tr>
<td>DCM fraction</td>
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</tr>
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</tr>
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<tr>
<td>Roots extract</td>
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<td>15</td>
<td>60</td>
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</tbody>
</table>
4.2.5 Phytotoxic activity

Sample plant extracts as well as solvents fractions were tested against *Lemma minor* in order to evaluate its promoter or inhibitor effect on plant growth as presented in Table 4.12. Differents concentration of ten, hundred and one thousand $\mu$g/mL and paraquat (standard drug) at concentration of 0.015 $\mu$g/mL were used for their phytotoxic effect. *B. Procumbens* extract showed moderate phytotoxic activity at various concentrations (20% at 10 $\mu$g/mL, 23% at 100 $\mu$g/mL and 30% at 1000 $\mu$g/mL, respectively). The roots extract inhibited the growth of *lemna* by 26%, 40% and 50% at the test concentration of ten, hundred and one thousand $\mu$g/mL, respectively. The leaf extract showed significant phytotoxic effect i.e. 50%, 60% and 70% at the same test concentrations. The DCM fraction showed excellent phytotoxic potential i.e. 50%, 63%, and 80% while hexane fraction showed mild activity i.e.13%, 20% and 26%) at the previous test concentrations.

<table>
<thead>
<tr>
<th>Samples</th>
<th>Conc. of sample (µg/ml)</th>
<th>No. of fronds Survived</th>
<th>No. of fronds Died</th>
<th>%Growth Regulation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Extract of sample plant</td>
<td>10</td>
<td>25</td>
<td>5</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>23</td>
<td>7</td>
<td>23</td>
</tr>
<tr>
<td></td>
<td>1000</td>
<td>21</td>
<td>9</td>
<td>30</td>
</tr>
<tr>
<td>n-Hexane fraction</td>
<td>10</td>
<td>26</td>
<td>4</td>
<td>13</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>24</td>
<td>6</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>1000</td>
<td>22</td>
<td>8</td>
<td>26</td>
</tr>
<tr>
<td>DCM fraction</td>
<td>10</td>
<td>15</td>
<td>15</td>
<td>50</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>11</td>
<td>19</td>
<td>63</td>
</tr>
<tr>
<td></td>
<td>1000</td>
<td>6</td>
<td>24</td>
<td>80</td>
</tr>
<tr>
<td>EtOAc fraction</td>
<td>10</td>
<td>24</td>
<td>6</td>
<td>25</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>22</td>
<td>8</td>
<td>36</td>
</tr>
<tr>
<td></td>
<td>1000</td>
<td>18</td>
<td>12</td>
<td>40</td>
</tr>
<tr>
<td>Leaf extract</td>
<td>10</td>
<td>15</td>
<td>15</td>
<td>50</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>12</td>
<td>18</td>
<td>60</td>
</tr>
<tr>
<td></td>
<td>1000</td>
<td>9</td>
<td>21</td>
<td>70</td>
</tr>
<tr>
<td>Roots extract</td>
<td>10</td>
<td>22</td>
<td>8</td>
<td>26</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>18</td>
<td>12</td>
<td>40</td>
</tr>
<tr>
<td></td>
<td>1000</td>
<td>15</td>
<td>15</td>
<td>50</td>
</tr>
</tbody>
</table>
4.2.6 Anti-leishmanial activity

The isolated compounds (1, 2, 4, 5 and 6) of *B. Procumbens* were screened for their leishmanicidal effect (promastigotes of *L. major*) as presented in Table 4.13 & figure 4.6. Compounds 5 and 4 showed significant antileishmanial activity with \( \text{IC}_{50} \) values of 18.37 ± 0.07 μM and 48.19 ± 0.06 μM. The other compounds such as 6 and 2 also exhibited considerable antileishmanial activity with \( \text{IC}_{50} \) values of 73.07 ± 0.06 μM and 85.12 ± 0.04 μM. The \( \text{IC}_{50} \) value of compound 1 was found more than hundred, which mean that the test compound is free of any leishmanicidal potential. Moreover, compounds 2, 4, 5 & 6 were less potent relative to the standard antileishmanial compound i.e. Pentamidine (\( \text{IC}_{50} \): 15.07 ± 0.06 μM).

**Table 4.13 Anti-leishmanicidal activity of isolated compounds of *B. procumbens***

<table>
<thead>
<tr>
<th>Code</th>
<th>Test organism</th>
<th>Compounds name</th>
<th>( \text{IC}_{50} ) (μM) ± S.E.M</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td></td>
<td>Indole-3-carbaldehyde</td>
<td>150.81 ± 0.09</td>
</tr>
<tr>
<td>2</td>
<td></td>
<td>2-hydroxybenzoic acid</td>
<td>85.12 ± 0.04</td>
</tr>
<tr>
<td>4</td>
<td><em>L. major</em> (DESTO)</td>
<td>Oleracein E</td>
<td>48.19 ± 0.06</td>
</tr>
<tr>
<td>5</td>
<td></td>
<td>Methyl 3, 4-dihydroxybenzoate</td>
<td>18.37 ± 0.07</td>
</tr>
<tr>
<td>6</td>
<td></td>
<td>Eupalitin-3-O-D-galactopyranoside</td>
<td>73.07 ± 0.06</td>
</tr>
<tr>
<td>Std</td>
<td></td>
<td>Pentamidine</td>
<td>15.07 ± 0.06</td>
</tr>
</tbody>
</table>

![Figure 4.6](image-url)  
*Figure 4.6 Leishmanicidal inhibition of the isolated compounds of *B. procumbens*
4.2.7 Acetylcholinesterase inhibitory assay

Table 4.14 shows the results of acetylcholinesterase (AChE) inhibitory activities of the isolated compounds in comparison with standard drug, Galanthamine. Among the compounds, Oleracein E (4) and Eupalitin-3-O-D-galactopyranoside (6) depicted considerable inhibition of acetylcholinesterase enzyme with IC$_{50}$ values of 54.39 ± 0.03 μM and 74.07 ± 0.06 μM. The compounds 1 and 5 showed no appreciable inhibition because their IC$_{50}$ values were more than 100.

**Table 4.14 Acetylcholinesterase inhibition of isolated compounds of B. procumbens**

<table>
<thead>
<tr>
<th>Code</th>
<th>Name of isolated compounds</th>
<th>IC$_{50}$ (μM) ± S.E.M</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Indole-3- carbaldehyde</td>
<td>135.17 ± 0.05</td>
</tr>
<tr>
<td>4</td>
<td>Oleracein E</td>
<td>54.39 ± 0.03</td>
</tr>
<tr>
<td>5</td>
<td>Methyl 3, 4-dihydroxybenzoate</td>
<td>104.37 ± 0.07</td>
</tr>
<tr>
<td>6</td>
<td>Eupalitin-3-O-D-galactopyranoside</td>
<td>74.07 ± 0.06</td>
</tr>
<tr>
<td>Standard</td>
<td>Galanthamine</td>
<td>0.5 ± 0.01</td>
</tr>
</tbody>
</table>

4.2.8 T cell proliferation assay

Five of the isolated compounds of *B. Procumbens* were screened for T cell proliferation assay (table 4.15). Compound 4 was found highly active with percent proliferation of 83.51 while compound 1 and 6 with percent proliferation of 32.02 and 24.1 respectively. Compound 2 and 5 were found fairly inactive in the assay, while PMA was used as control.

**Table 4.15 T cell proliferation assay of compounds**

<table>
<thead>
<tr>
<th>Code</th>
<th>Concentration</th>
<th>*Treg cells Proliferation (% increase)</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>100 μM</td>
<td>32.02</td>
<td>Active</td>
</tr>
<tr>
<td>2</td>
<td>100 μM</td>
<td>--</td>
<td>Inactive</td>
</tr>
<tr>
<td>4</td>
<td>100 μM</td>
<td>83.51</td>
<td>Highly active</td>
</tr>
<tr>
<td>5</td>
<td>100 μM</td>
<td>--</td>
<td>Inactive</td>
</tr>
<tr>
<td>6</td>
<td>100 μM</td>
<td>24.18</td>
<td>Active</td>
</tr>
<tr>
<td>*PMA</td>
<td>100 μM</td>
<td>60.15</td>
<td>Active</td>
</tr>
</tbody>
</table>

* PMA (Standard): Phorbol myristate acetate and *Treg: CD4+CD25+ regulatory T cells.
4.2.9 Cytotoxic activity (NIH-3T3 cell lines)

Table 4.16 shows the results of cytotoxicity effect of six compounds against NIH 3T3 cell lines. Compound 4 showed less cytotoxicity with IC$_{50}$ value of 38.81 ± 2.93 μM at the test concentration of 100 μM. The IC$_{50}$ value of the other compounds was found more than 100, which mean that these compounds are non cytotoxic in their effect against NIH-3T3 cell lines.

<table>
<thead>
<tr>
<th>Code</th>
<th>Concentration</th>
<th>IC$_{50}$ μM</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>100 μM</td>
<td>&gt;100 ± 1.42</td>
<td>Non cytotoxic</td>
</tr>
<tr>
<td>2</td>
<td>100 μM</td>
<td>&gt;100 ± 1.66</td>
<td>Non cytotoxic</td>
</tr>
<tr>
<td>4</td>
<td>100 μM</td>
<td>38.81 ± 2.93</td>
<td>Less cytotoxic</td>
</tr>
<tr>
<td>5</td>
<td>100 μM</td>
<td>&gt;100 ± 1.74</td>
<td>Non cytotoxic</td>
</tr>
<tr>
<td>6</td>
<td>100 μM</td>
<td>&gt;100 ± 1.84</td>
<td>Non cytotoxic</td>
</tr>
</tbody>
</table>

4.3 In-vivo Biological activities

4.3.1 Acute toxicity

Compound 6 and crude extract of sample plant were screened for their toxic effect at different doses as presented in Table 4.17. Crude extract (2000 mg/kg) and compound 6 (500 mg/kg) were found no sign of toxicity at the respective doses. After 24 hrs evaluation time, all the animals were alive and no signs of considerable mortality were observed in the animals. So we concluded that the test dose of samples was nontoxic and can be used for further experimental work.

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Dose (mg/kg)</th>
<th>No. of animal alive after 24 hrs</th>
<th>No. of animal dead after 24 hrs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Compound 6</td>
<td>50</td>
<td>All</td>
<td>Nil</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>All</td>
<td>Nil</td>
</tr>
<tr>
<td></td>
<td>300</td>
<td>All</td>
<td>Nil</td>
</tr>
<tr>
<td></td>
<td>500</td>
<td>All</td>
<td>Nil</td>
</tr>
<tr>
<td>C.extract of BP plant</td>
<td>500</td>
<td>All</td>
<td>Nil</td>
</tr>
<tr>
<td></td>
<td>1000</td>
<td>All</td>
<td>Nil</td>
</tr>
<tr>
<td></td>
<td>2000</td>
<td>All</td>
<td>Nil</td>
</tr>
<tr>
<td>Normal saline</td>
<td>10</td>
<td>All</td>
<td>Nil</td>
</tr>
</tbody>
</table>
The acute toxicity is evaluated to find out the toxic dose or any adverse behaviour observed during the procedure. It is also important to be evaluated before any other in-vivo assay, as to find out the safe dose. The reason for death observed during any other protocol can be subsided from the toxicity caused by extract.

4.3.2 Acetic acid induced writhing assay

*B. Procumbens* extract showed significant antinociceptive effect as compared to control (normal saline) (Table 4.18). Methanolic crude extract (400 mg/kg) exhibited significant effect (p<0.01) against acetic acid induced paw. The percent inhibition at various doses of extract was 21.19% at 100 mg/Kg, 61.31% at 200 mg/Kg, and 73.07% at 400 mg/Kg, respectively. However, the diclofenac sodium (standard drug) at a dose of 10 mg/Kg produced maximum percent inhibition (83.85%) of pain, which is greater than the any effect of the tested dose of crude extract of *B. Procumbens* plant.

**Table 4.18 Analgesic effect (acetic acid induced writhing model) of crude extract**

<table>
<thead>
<tr>
<th>Sample</th>
<th>Dose (i.p.)</th>
<th>No. of writhings (10 min.)</th>
<th>% inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline</td>
<td>10 ml/Kg</td>
<td>63.92 ± 3.38</td>
<td></td>
</tr>
<tr>
<td>Extract</td>
<td>100 mg/Kg</td>
<td>50.37 ± 2.41</td>
<td>21.19</td>
</tr>
<tr>
<td></td>
<td>200 mg/Kg</td>
<td>24.73 ± 2.89</td>
<td>61.31*</td>
</tr>
<tr>
<td></td>
<td>400 mg/Kg</td>
<td>17.21 ± 2.34</td>
<td>73.07**</td>
</tr>
<tr>
<td>Diclofenac Na</td>
<td>10 mg/Kg</td>
<td>10.32 ± 1.19</td>
<td>83.85**</td>
</tr>
</tbody>
</table>

Values are reported as mean ± Standard error of mean (n = 6). Analysis of variance (ANOVA) followed by Dunnett’s test was applied to determine statistical significance.*P < 0.05, **P < 0.01.

4.3.3 Hot plate induced pain model

The central analgesic effect of *B. Procumbens* extract was observed as an increase in latency time in seconds that was noted at every interval of time of 30 min from 0-120 min after the administration of saline, samples and Tramadol (Table 4.19). Extract (400 mg/kg) showed more significant effect (p < 0.01) after sixty min of administration that remained significant till 120 min. Tramadol (standard drug) showed significant effects after 60 min of administration which remained significant till 120 min.
Table 4.19 Analgesic effect of crude extract in hot plate induced pain model

<table>
<thead>
<tr>
<th>Sample</th>
<th>Dose (mg/Kg)</th>
<th>Latency of nociceptive response (sec)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>Saline</td>
<td>-</td>
<td>8.31 ± 0.22</td>
</tr>
<tr>
<td>Extract</td>
<td>100</td>
<td>8.32 ± 0.27</td>
</tr>
<tr>
<td></td>
<td>200</td>
<td>8.30 ± 0.31</td>
</tr>
<tr>
<td></td>
<td>400</td>
<td>8.31±0.27</td>
</tr>
<tr>
<td>Tramadol</td>
<td>20</td>
<td>8.44 ± 0.29</td>
</tr>
</tbody>
</table>

Mean ± Standard error of mean (n = 6). Analysis of variance (ANOVA) followed by Dunnett’s test was applied to determine statistical significance.*P<0.05, **P<0.01.

4.3.3 Anti-inflammatory activity

*B. Procumbens* extract showed significant anti-inflammatory effect when compared to control and results are summarized in Table 4.20a-4.20b. Crude extract (400 mg/kg) showed significant anti-inflammatory effect (p<0.01) after 3 hrs of carrageenan administration and this condition remained significant till 5 hours. Similarly, the same dose also showed maximum percent inhibition (47.98%) of paw edema at 5 hrs of observation. The percent inhibitory effect of indomethacin (69.31%) was greater than that of the highest dose of our test extract. The anti-inflammatory effect of extract was in dose-dependent manner.

Table 4.20a Anti-inflammatory activity of crude extract

<table>
<thead>
<tr>
<th>Sample</th>
<th>Doses (mg/kg)</th>
<th>Increase in paw edema size (mm) ± S.E.M</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>3rd hour</td>
</tr>
<tr>
<td>Saline</td>
<td>-</td>
<td>5.73 ± 1.42</td>
</tr>
<tr>
<td>Extract</td>
<td>100</td>
<td>5.54 ± 1.26</td>
</tr>
<tr>
<td></td>
<td>200</td>
<td>4.65 ± 0.73*</td>
</tr>
<tr>
<td></td>
<td>400</td>
<td>3.58±0.47**</td>
</tr>
<tr>
<td>Indomethacin</td>
<td>10</td>
<td>3.05±0.69**</td>
</tr>
</tbody>
</table>

Mean ± Standard error of mean (n = 6). Analysis of variance (ANOVA) followed by Dunnett’s test was applied to determine statistical significance.*P<0.05, **P<0.01.
Table 4.20b Percent inhibition edema of the crude extract of *B. Procumbens*

<table>
<thead>
<tr>
<th>Sample</th>
<th>Doses (mg/kg)</th>
<th>% inhibition edema</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>3&lt;sup&gt;rd&lt;/sup&gt; hour</td>
</tr>
<tr>
<td>Saline</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Extract</td>
<td>100</td>
<td>3.31</td>
</tr>
<tr>
<td></td>
<td>200</td>
<td>18.85</td>
</tr>
<tr>
<td></td>
<td>400</td>
<td>37.52</td>
</tr>
<tr>
<td>Indomethacin</td>
<td>10</td>
<td>46.77</td>
</tr>
</tbody>
</table>

4.3.4 Hepatoprotective activity

4.3.4.1 Effect of Eupalitin-3-O-D-galactopyranoside (EGP) compound on biochemical parameters

Table 4.21a show the five groups of animals which includes vehicle control, toxic, standard and isolated compound of *B. Procumbens* in the experimental design of hepatoprotective activity. The results of hepatoprotective activity of the EGP compound in CCl<sub>4</sub> intoxicated rats have been shown in Table 4.21b. The serum levels of SGPT, SGOT, ALP and total bilirubin were significantly increased (64 ± 1.89 U/ml, 86 ± 1.47 U/ml, 252.6 ± 2.96 U/ml and 5.45 ± 0.32 mg/dl, respectively) in group treated with CCl<sub>4</sub> alone when compared with control. However, simultaneous administration of EGP compound at dose of 60 mg/kg with CCl<sub>4</sub> significantly (p<0.01) reduced the levels of SGPT and SGOT (26 ± 1.34 U/ml and 42.92 ± 1.6 U/ml) enzymes when compared with the group treated with CCl<sub>4</sub> alone (23.85 ± 1.72 U/ml and 34.53 ± 2.41U/ml). In case of ALP and total bilirubin (179.22 ± 3.41U/ml and 3.23 ± 0.19 mg/dl), significant (p<0.01) decline was observed in group treated with 60 mg/kg dose. The low dose i.e. 30 mg/kg produced non-significant decline in SGOT, SGPT, ALP and total bilirubin. 60 mg/kg dose showed significant hepatoprotective activity with a maximum reduction in the serum enzyme levels, however, it was not effective as silymarin. Silymarin (standard drug) also reversed the hepatotoxicity significantly decrease the serum levels of SGPT, SGOT and ALP (23.85 ± 1.72, 34.53 ± 2.41and 146.89 ± 1.34 U/ml, respectively).
Table 4.21a Experimental design of hepatoprotective activity

<table>
<thead>
<tr>
<th>Groups</th>
<th>Design of treatment</th>
<th>Dose (mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group I</td>
<td>Control</td>
<td>Liquid paraffin</td>
</tr>
<tr>
<td>Group II</td>
<td>CCl₄ only</td>
<td>2.5</td>
</tr>
<tr>
<td>Group III</td>
<td>Silymarin + CCl₄</td>
<td>25</td>
</tr>
<tr>
<td>Group IV</td>
<td>EGP compound + CCl₄</td>
<td>30</td>
</tr>
<tr>
<td>Group V</td>
<td>EGP compound + CCl₄</td>
<td>60</td>
</tr>
</tbody>
</table>

Table 4.21b Effects of the EGP of *B. Procumbens* on biochemical parameters in CCl₄ induced hepatotoxicity in rats

<table>
<thead>
<tr>
<th>Treatment</th>
<th>SGPT(U/ml)</th>
<th>SGOT(U/ml)</th>
<th>ALP(U/ml)</th>
<th>TB (mg/dl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>27.14 ± 1.81</td>
<td>29.11 ± 1.2</td>
<td>114 ± 2.93</td>
<td>1.23 ± 0.17</td>
</tr>
<tr>
<td>CCl₄ only</td>
<td>64 ± 1.89</td>
<td>86 ± 1.47</td>
<td>252.6 ± 2.96</td>
<td>5.45 ± 0.32</td>
</tr>
<tr>
<td>Silymarin + CCl₄</td>
<td>23.85 ± 1.72**</td>
<td>34.53 ± 2.41**</td>
<td>146.89 ± 1.34**</td>
<td>2.45 ± 0.78**</td>
</tr>
<tr>
<td>EGP comp + CCl₄</td>
<td>53.26 ± 1.57</td>
<td>71.4 ± 2.33</td>
<td>237.3 ± 2.78</td>
<td>4.73 ± 0.21</td>
</tr>
<tr>
<td>EGP comp + CCl₄</td>
<td>26 ± 1.34**</td>
<td>42.92 ± 1.6**</td>
<td>179.22 ± 3.41*</td>
<td>3.23 ± 0.19*</td>
</tr>
</tbody>
</table>

Data are mean ± Standard error of mean (n = 6)
*p<0.05, **p<0.01: Groups IV to V are compared with group III.
Analysis of variance (ANOVA) followed by Dunnet’s t-test.

4.3.4.2 Histopathological studies

Histology investigation of liver tissues of control group showed normal hepatocytes with dilation in sinusoids and well brought out central vein in Fig 4.7. Group treated with CCl₄ alone showed congestion of the central vein with aggregation of lymphocytes around the central vein. The necrosis of hepatocytes and inflammatory cells infiltration with sinusoids congestion were observed in Fig 4.8. Mice treated with CCl₄ (intoxicated) and standard drug silymarin showed less disarrangement and degeneration of hepatocytes cell and sinusoids, indicating marked regeneration of the cellular architecture of liver in Fig 4.9. EGP compound was used in this experiment at dose of 30 mg/kg and 60 mg/kg followed by a simultaneous treatment with CCl₄. 60 mg/kg
dose showed significant effect than 30 mg/kg dose in regeneration of hepatocytes and sinusoids as showed from a considerable reduction in necrosis Fig 4.10.

Figure 4.7 Control group: Cross section in liver showing the normal structure of central vein (large arrow), hepatocytes (small arrow) and sinusoids (arrow head).

Figure 4.8 CCl₄ treated rats: Showing congestion of the central vein (arrow head). The necrosis of hepatocytes (large arrow) and congestion sinusoids (small arrow).
Figure 4.9 CCl₄ + Silymarin: Cross section in liver showing the restored structure of hepatocytes cell (large arrow) and sinusoid space (small arrow)

Figure 4.10 EGP comp (30 mg/kg) + CCl₄ (Left slide) and EGP comp (60 mg/kg) + CCl₄ (Right slide): The cross section in liver of both sildes are showing the normal structure of hepatocytes (large arrow) and sinusoids (small arrow)
5. DISCUSSIONS

5.1 Chemical composition

5.1.1 Proximate composition

In the proximate analysis, nitrogen-free extract (NFE) was found significant amount in aerial parts which is a main source of energy (Mensah et al., 2008). Other nutrients such as protein and inorganic matter (ash) were also recorded in highest concentration in the roots. The protein content can contribute to the daily protein requirement (NRC, 1975) whereas; ash content could be a good source of minerals. Fiber content was found high in aerial parts of *B. Procumbens* that may help in the prevention of chronic diseases (Diabetes mellitus, cardiovascular, cancer etc) (Bowman and Russell, 2001). Our results of NFE and protein concentration of aerial parts and roots of sample plant were found higher than the reported values of NFE and protein of *B. diffusa* by Juna Beegum et al., (2014). In another study Puranik et al., (2012) evaluated the value of moisture (76.04 %) and carbohydrates (17.14 %) which were found to be in higher concentration than other nutrients of the *B. diffusa*.

5.1.2 Mineral composition

The reported minerals in the aerial parts and roots of *B. Procumbens* may account for the ethno-medicinal use of the plant in the remedy of many diseases. The physiological function of the body such as muscular function, maintenance of strong bones and synthesis of enzymes mainly depends on the daily recommended intake of calcium and potassium. In this study, the calcium and potassium contents were found in highest amount in aerial parts, which can be used for normal body functions and prevention of osteoporosis disease (Aliyu et al., 2008; Wallace et al., 2004). Similarly, the Iron content was also found in highest level in roots indicating that it can be used to improve the anaemic condition of a patient (Akpabio and Ikpe, 2013). The recommended value of Na/K ratio of hypertensive patients is 0.6 while the values of aerial parts and roots of sample plant were found lower (0.11 and 0.32) than the recommended value (Nieman et al., 1992). Therefore, studied plant may have beneficial effects on hypertensive patients. The concentration of lead was lower than the maximum permissible limit (0.3 ppm) whereas cadmium was not detected in the sample plant (WHO, 1998). So this
plant is nontoxic for human consumption on the basis of above results. Abd EI-Salam et al., (2013) reported higher values of iron and zinc in various parts (roots, stem and leaves) of *B. Procumbens* than the results of our study. In another study puranik et al., (2012) evaluated the fresh leaves of *B. diffusa* for phosphorous (151.45 mg/100 g), sodium (160.21 mg/100 g), and magnesium contents (8.93 mg/100 g). They found higher values except calcium (218.24 mg/100g) which found in lower concentration than the present study.

5.1.3 Amino acids composition

Aspartic acid and glutamic acid in the aerial parts of sample plant were found in highest amount and can be used to maintain the cognitive functions of the brain (Leon, 1986; McEntee and Crook, 1993). It is suggests that aerial part can be used for improving neurotransmitter function and as stabilizing agent for brain. High phenylalanine was also found in roots which have been involved in the biosynthesis of phenolic compounds (Hass, 1975). Other interesting amino acids such arginine and tryptophan was found in high amount in aerial parts of *B. Procumbens*. Arginine has good antioxidant activity (Boger et al., 1996) and also necessary for children growth (Robinson, 1987), while tryptophan produces serotonin. Serotonin is mood enhancing hormone and its decrease level causes depression (Akram et al., 2011). There are very limited data available regarding the analysis of amino acid profile of sample plant. Hence, the amino acid composition is reported for the first time from *B. Procumbens* plant.

5.2 Qualitative and quantitative analysis of phytochemicals

Methanol extract and EtOAc solvent fraction confirms the presence of cardiac glycosides and flavonoids. Phenolic contents are the major plant metabolites as well as showed significant radical scavenging ability (Tosun et al., 2009). Similarly, flavonoids have been showed considerable chelating properties and to suppress carcinogenesis in various animal models (Shariffifar et al., 2008; Manthey et al., 2001). Phenolic and flavonoid metabolites possess diverse biological activities such as anti-carcinogenic, anti-atherosclerotic, anti-inflammatory and radical scavenging properties (Chung et al., 1998; Miliauskas et al., 2004). Our results of phenolic and flavonoids contents were
found higher as compared to those reported by Bokhari, *et al.*, (2015), Abbasi *et al.*, (2012) and Juna Beegum *et al.*, (2014).

### 5.3 Radical scavenging activity

The ethyl acetate solvents fractions of sample plant showed strong radical scavenging activity. This activity was probably due to the presence of flavonoids (Heim *et al.*, 2002; Mensor *et al.*, 2001). The antioxidant activity has been checked with different methods but the most widely used methods are those to generate free radical and then neutralized by antioxidant compounds (Arnao *et al.*, 2001). In this case, DPPH model are the standard method and has been used for the evaluation of anti-radical activity (Sanchez-Moreno *et al.*, 1998). The findings of the present study are also in close agreement with that of Abbasi *et al.*, (2012) who reported that EtOAc fraction of *B. procumbens* has maximum value of DPPH free radical scavenging (82.54 %) while the IC$_{50}$ value of 37.11μg/ml. The results of the present study showed higher IC$_{50}$ values for free radical scavenging than those reported by Bokhari *et al.*, (2015) for *B. procumbens* methanol extract (78.3 μg/ml).

### 5.4 Antimicrobial activity

Recently, the irrational use of antibiotic have increased immediately, which have led to the development and dissemination of multi-drug resistant strains of several groups of microorganisms (WHO, 2001, Aibinu *et al.*, 2003; Aibinu *et al.*, 2004). Moreover, the non-availability and high cost of antibiotics have resulted in increase the morbidity and mortality due to the non-availability and high cost of new generation antibiotics (Williams, 2000). Thus, plant extracts, different solvent fractions and isolated compounds are still valuable antimicrobials which are mostly used as source of lead compounds for the synthesis of an array of antimicrobials (Linton, 1983).

Our results indicate that dichloremethane and *n*-hexane solvents fractions followed by EtOAc fraction are more effective in inhibiting the growth of pathogenic bacterial and fungal strains. In the present investigations, the antibacterial activity of crude extract and fractions of *B. Procumbens* against phytopathogenic bacteria such as *C. michiganensis* and *X. campestris* are reported for the first time. *X. campestris* causes the rice blight, cabbage black rot, and citrus blight diseases around the world (Britto *et al.*, 2001).
F. oxysporum is also important from agriculture point of view as it causes *Fusarium* wilt disease in tomato plant (Ramaiah and Garampalli, 2015). Crude extract and fractions of studied plant can be used in the management of these diseases caused by the above mentioned bacterial and fungal strains. The methanol soluble fraction of *B. repens* displayed higher activity against *S. aureus* (12.2 mm), *B. subtilis* (11.8 mm) and *E. coli* (16.7 mm) than the methanol leaf extract of BP (Rahman *et al.*., 2014).

### 5.5 Brine shrimp lethality bioassay

Brine shrimp is a reliable and general bioassay for the screening of plant extracts, fractions and isolated compounds of medicinal plants for pharmacological activity. Furthermore, this assay is the most useful tools for the preliminary assessment of general toxicity (Solís *et al.*., 1993). The bioactivity of medicinal plants has been determined using this assay because it is more safe, practical and economical method (Subhan *et al.*., 2008). Bussmann *et al.*., (2011) classified crude extracts/fractions and pure substances into highly toxic (LD$_{50}$ < 249 μg/ml), moderate toxic (LD$_{50}$ < 250-499 μg/ml), low toxic (LD$_{50}$ < 500-1000 μg/ml) and non-toxic (LD$_{50}$ >1000 μg/ml).

Based on this, the results of our study showed that the dichloremethane (DCM) fraction was toxic while roots extract was moderately toxic. The results of our experiment are supported by the findings of Gautam *et al.*., (2016) who reported the LD$_{50}$ value of methanol extract of *B. diffusa* was found to be 165.19 μg/mL. In another study, Apu *et al.*., (2012) showed that n-hexane extract of *B. diffusa* has LD$_{50}$ value of 140.55 μg/mL.

### 5.6 Larvicidal Bioassay

Larviciding in breeding places of mosquito is one of the successful ways of reducing mosquito densities. Larviciding is most commonly performed with synthetic insecticide such as pyrethroids, organophosphates, organochlorines, carbamates, insect growth regulators etc. (Hedlin, *et al.*., 1997). Mosquitos are the most common vectors of many vector-borne diseases related to humans and other animals (Su and Mulla, 1998). Among the mosquitos, species belonging to *Anopheles, Aedes* and *Culex* genera are vectors for the parasites causing malaria, Japanese encephalitis, filariasis, dengue fever and yellow fever (Sukumar et al., 1991). *A. aegypti* and *C. quinquefasciatus* are the most common species in mosquito’s family. *C. quinquefasciatus*, which is a vector of
lymphatic filariasis (LF), found world-wide mostly in temperate regions. Annually about 120 million cases of LF are reported worldwide with 44 million people having lymph edema of the breast, upper or lower limbs, genitals, or hydrocele, the latter being the most frequent chronic manifestation (Bernhard et al., 2003).

Plants, which are natural sources of different compounds, are recognized to contain larviciding, adulticidal, and repellency agents that may act synergistically or individually. Some phytochemicals act as general toxicants effective against more than one life cycle stages of mosquitoes, while others act as repellents or attractants (Ghayal, et. al., 2010). Natural products are best option because it is less harmful to environment and non-targeted organisms. Several extracts/fractions and compound from different plants families have been evaluated for new and promising larvicides (Ester, et. al., 2008).

In our study the extracts and solvents fractions of sample plant in various concentrations, produced significant mortalities in 3rd & 4th instars larvae of C. quinquefasciatus after 24 hrs of exposure. The application of leaf extract was highly toxic that resulted in maximum larval mortalities. Leaf extract proved superior to the other samples against 3rd & 4th instars larvae of C. quinquefasciatus after 24 hrs exposure periods. In this assay, the LC50 values were found minimum for leaf extract as well as hexane fraction indicating high toxicity against the pesticidal action of mosquito species. The roots extract gave maximum LC50 value exhibited low toxicity against the killing of mosquito species. This bioassay is reported for the first time from B. procumbens plant.

5.7 Phytotoxic activity

Lemna plants are sensitive to a number of pollutants, making it advantageous in toxicity assessment (Ateeq-ur-Rehman et al., 2009). In addition, it is also reported that natural antitumor compounds inhibit the growth of this plant while some substances might trigger frond growth; hence, this assay is also useful for the detection of novel plant growth stimulants (Atta-ur-Rehman, 1991). Thus fulfilling the modern-day demand of natural, biodegradable herbicides and plant growth stimulants (Cayuela et al., 2007; Wang, 1990 and Lewis, 1995).
Various pesticides (weedicides and herbicides) are employed with the aim to effectively control weeds and increase crop yield (Kim, 1994; Santos, 2009). However, the indiscriminate uses of synthetic herbicides lead sometimes to an increased risk of herbicide resistant weed biotypes (Heap, 2014), environmental pollutions (Aktar et al., 2009; Pell et al., 1998 and Roger et al., 1994) and prolong persistence like DDT (Clarkson, 1995 and Snelder et al., 2008). In this respect, alternative weed management strategies by natural weedicides that are biodegradable, ecofriendly and cost-effective are therefore a time demanding issue throughout the world. It is thus necessary to study and explore the phytotoxic activity of plants and their compounds for the development of natural plant growth regulators or biological herbicides (Morimoto et al., 2009).

In this activity, DCM solvent soluble fraction showed effective inhibition against the *lemna* plant growth. So this solvent fraction may be used for effective natural herbicides for controlling weeds in organic farming. This activity is also conducted for the first time from *B. procumbens*.

### 5.8 Anti-leishmanicidal activity

Leishmaniasis is considered as a major health problem, having significant mortality and morbidity rate across the world. It is endemic in 88 countries among them 72 are developed and 13 are least developed countries. Majority of cases are from Pakistan, Brazil, Algeria, Peru, Saudia Arabia and Syria (David and Craft, 2009). In Pakistan highest disease effected regions are tribal areas of KPK and Southern province of Sindh in the district Larkana and Dadu (Ullah et al., 2009). Some cases of cutaneous leishmaniasis were also reported in Karachi, maximum cases were observed in district Malir, lyari and central areas of the city (Sheikh et al., 2003) and some cases observed in Balochistan (gazozai et al., 2010). Cutaneous leishmaniasis is the most commonly prevalent form of Leishmania infections having an estimated incidence range of 0.7 to 1.2 million cases each year (Alvar et al., 2012).

The treatment options for leishmaniasis include potentially toxic heavy metal-containing compounds, such as pentavalent antimonials and others. Despite toxic nature, these compounds also need to be administered over prolonged period of times and possess serious adverse effects at therapeutic dose ranges, such as pancreatitis, cardiotoxicity and musculoskeletal disorders. Amphotericin B and pentamidine are
alternative treatment options for leishmaniasis, however, these drugs are also associated with adverse effects involving multiple organs including renal toxicity, bone marrow suppression, and disturbance of glucose metabolism (Croft and Coombs, 2003; Chan-Bacab and Pena-Rodriguez, 2001 and Osorio et al., 2007).

Natural products are a potential source of new drugs for the treatment of many diseases, including leishmaniasis. Furthermore, it has been listed over 200 plant products that have shown antileishmanial properties (Singh et al., 2014). Results indicate that some of the isolated compounds of B. Procumbens have favorable leishmanicidal activity and especially the methyl 3, 4-dihydroxybenzoate compound which may be develop as new and potential leishmanicidal agent. This is for the first time that leishmanicidal activity of sample plant is reported.

5.9 Acetylcholinesterase inhibitory assay

Some of the plant extracts are already used in the treatment of various diseases such as insomnia, amnesia, depression and anxiety or to prolong longevity, improved memory and cognitive function (Houghton and Howes, 2003). The investigation of the effects of active compounds extracted from plants is necessary, since they may provide new insights into the clinical treatment of neurodegenerative diseases. A number of researchers have evaluated the AChE inhibition activity of several plant species, (Adersen et al., 2006; Fleming, 1981; Dieu et al., 2005). Some studies indicate drugs derived from natural products as inhibitors of AChE, which would make them effective in the treatment of AD for example, Galanthamine (alkaloid from snowdrop tree), was recently approved for AD (Ingkaninan et al., 2003; Heinrich and Teoh, 2004). These research studies were conducted with the intention of discovering new and potentially safe molecules against AD. In this regards, it is concluded from the present study that compound (4) of B. Procumbens showed best inhibition against AChE and may help in preventing Alzheimer disease. This activity is also reported for the first from this plant.

5.10 Treg cell proliferation assay

Auto-immune diseases such as arthritis, inflammation of joints, skin infections, brain and other organ triggered by abnormal immune responses can be cured by using targeted immun-suppressant therapy (Shevach, 2009). Moreover, these diseases also
increase due to the reduced number of NK cell/cytolytic activity of immune system (Lotzová, 1991). Some medicinal plants have shown effective immune modulatory activity as well as possess potential biological agents to treat immune disorders (Actor and Dasgupta, 2003; Clement-Kruzel et al., 2008). The findings of the present study are in close agreement with that of Pandey et al., (2005), who reported that Eupalitin-3-O-D-galactopyranoside compound significantly suppressed the PHA-stimulated proliferation of human peripheral blood mononuclear cells from 63-98% at the concentration of 500 μg/ml. From the current study it is clear that compound (4) of studied plant can be used as an increas from the Treg cell proliferation.

5.11 Cytotoxicity activity (NIH-3T3 cell lines)

*In-vitro* cytotoxicity assays are generally used to discover the toxicity of a compound in humans, and to generally identify toxic products (Clemedson and Ekwall, 1999; Scheers and Dierickx, 2001). Newely discovered compounds should be screened for their cytotoxic potential before going to *in-vivo* procedures. The cytotoxic potential of samples has been evaluated using 3-(4,5-dimethylthiazol-2-yl)-2,5- diphenyltetrazolium (MTT) assay (Smee et al., 2002). Compounds with least inhibitory potential in the mentioned experiment are claimed to be safe. Hence it is concluded that compound (4) was found less cytotoxic while, remaining isolated compounds of *B. Procumbens* was found non cytotoxic and can be implicated in *in-vivo* protocols.

5.12 *In-vivo* analgesic activity

The peripheral and central analgesia of the crude extract of studied plant were evaluated in the current study using two models, namely chemical visceral (acetic acid induced) and thermal (hot plate). Acetic acid-induced writhing model is a well recommended protocol for screening of analgesic drugs (Raquibul et al., 2010). It has been used to screen peripherally acting analgesics (Gené et al., 1989). In this model, the acetic acid produces writhing reflex in animals by activating the chemo sensitive nociceptors (Onasanwo and Elegbe, 2006). Furthermore, acetic acid causes pain by releasing endogenous inflammatory mediators (cyclooxygenase & prostaglandin) (Taesotikul et al., 2003; Mazid et al., 2010). The hot plate model has been used to study centrally acting analgesics (Woolfe and MacDonald, 1994) as sensory nerves sensitise the nociceptors and the involvement of endogenous substances such as
prostaglandins are minimized (Bachlav et al., 2009). Our results are fairly in line with Hiruma-Lima et al., (2000) who tested the lyophilized decoction and fresh leaf juice of B. diffusa using acetic acid induced writhing and hot plate models. The dipyrone sodium (200 mg/kg) was used as standard drug. The leaf juice and lyophilized decoction were used at a dose (p.o) of 1000 mg/kg. Leaf juice produced a significant increase in latency during the observation time while the lyophilized decoction, however, only raised the pain thresholds during the first 30 min of observation in the hot-plate model. For the first time, the analgesic activity is reported from B. procumbens in which the crude extract of this plant showed significant peripheral effect than central analgesic effect.

5.13 **In-vivo anti-inflammatory activity**

This assay is a well-established animal model to detect anti-inflammatory agents in natural products (Di Rosa et al., 1971). In the anti-inflammatory activity, the formation of edema in the paw of the rat has two phases (Vinegar et al., 1969). The first phase is ascribed to the release of histamine and serotonin (Maity et al., 1998) while the second phase involves the swelling due to release of prostaglandin like substance (Perez-Guerrero et al., 2001). The results of our study are in line with Bokhari et al., (2015) who stated that n-butanol and methanol extracts of B. procumbens significantly inhibited the edema after 1st, 2nd and 3rd hours when compared to the diclofenac potassium. The data of the present study are in close similarity with Nagarajaiah et al., (2013), who evaluated the aqueous root extract (1000 mg/kg) of B. diffusa and compared it, to the standard drug (valdecoxib, 5 mg/kg). The extract significantly inhibited (P <0.001) the carrageenan induced rat paw edema when compared to valdecoxib. The percent inhibitory effect of extract and valdecoxib was 52.67 % and 82.67 %, respectively, after 5 hrs of observation. B. Procumbens extract (400 mg/kg, i.p.) showed significant anti-inflammatory activity when compared to that induced by indomethacin.

5.14 **In-vivo hepatoprotective activity**

Carbon tetrachloride (CCL₄) is a common hepatotoxic agent used in the research studies of liver diseases (Johnson and Kroening, 1998). The variation occurred in permeability membrane of hepatocytes with subsequent leakage of hepatic enzymes including SGOT
and SGPT into the circulation due to toxic effect of CCl₄. Thus the elevated level of hepatic enzymes in the serum can be employed as a marker of hepatotoxicity assessment (Sallie et al., 1991). The enzyme AST is primarily found in the mitochondria of hepatocytes whereas, ALT, being more specific to the liver, acts as a better parameter for hepatic injury detection. In addition, elevation of serum ALP, total proteins as well as total bilirubins are also linked with liver injury and reflected the level of jaundice. In practice ALT, ALP and AST activity as well as serum total bilirubin levels are the widely used biochemical parameters for evaluation of liver injury. It is well understood that elevated serum levels of enzymes like ALP, SGOT, SGPT and bilirubin are due to deleterious effects of hepatotoxic agent on structural integrity of liver indicating hepatotoxicity (Saraswat et al., 1993). In the present study administration of Eupalitin-3-O-β-D-galactopyranoside (EGP) compound has decreased serum levels of marker enzymes like ALP, SGOT, SGPT and total bilirubin level. These results are in agreement with the commonly accepted view that serum ALP, SGOT and SGPT levels return to normal after healing of hepatic parenchyma and the regeneration of hepatocytes. Similar investigation has been reported by chakraborty and handa, (1989), where hepatoprotective activity of the isolated steroidal and flavone compounds (androst 5-ene analogue and 6, 5′-dimethoxy- 5, 7, 3′-trihydroxyflavone at concentration of 200 µg/ml & 50 µg/ml, respectively), from the aerial parts of B. diffusa against CCl₄ intoxication models has been evaluated. Venkatesh et al., (2000) and chandan et al., (1991) also reported the same studies and used crude extract of B. diffusa against CCl₄ induced hepatotoxicity in rats. However, in our study we used pure compound for hepatoprotective activity, while these studies used crude extract for this activity. This activity is reported for the first time on pure compound (EGP) of B. Procumbens which showed strong potential to be further investigated as a new lead compound for management of hepatotoxicity.

5.15 Structure elucidation of isolated compounds of B. Procumbens

5.15.1 Indole-3-carboxaldehyde

This compound is an active metabolite of plant and belongs to the derivatives of indole. This compound is reported for the first time from B. procumbens. Anderton et al., 2004 & Yue et al., (2000) reported that this compound is commonly present in microorganisms and plants and possess strong antimicrobial and antitumor activities.
Indole-3-carboxaldehyde is also potential source for fungi-derived pesticides for the control of plant diseases (Chen et al., 2012). Ashour et al., (2007) showed growth inhibition activity (53% cytotoxic activity at concentration 10.0 μg/ml) against the L5178Y cell line.

5.15.2 2-hydroxybenzoic acid (salicylic acids)

Salicylic acids are an effective plant metabolite. This compound is used as non-steroidal anti-inflammatory drug and well-known its ability to relieve the aches, pains, fevers (Madan and Levitt, 2014; Delaney, 2010). This compound can act as keratolytic agent (WHO, 1997) which is a key ingredient in many skin-care products (Madan and Levitt, 2014).

5.15.3 4-hydroxybenzoic acid

It is also known as p-hydroxybenzoic acid. This compound has been isolated from the leaves of E. heterophylla and exhibited good activity against the xanthine oxidase enzymes (Falodun et al., 2008). Moreover, 4-HBA has been isolated from various sources including carrots (Sircar and mitra, 2009), palm oil (Chong et al., 2009) and grapes (Ling et al., 2005). This compound also showed promising pharmacological activities such as antifungal, anti-mutagenic, anti-sickling, estrogenic (Pugazhendhi et al., 2005), and antimicrobial (Chong et al., 2009).

5.15.4 Oleracein E (tetrahydroisoquinoline)

It is a phenolic alkaloids compound and showed strong antioxidant property (Yang et al., 2009). In the current study, this compound is also isolated for the first time from B. procumbens. Oleracein E has been showed potent DPPH radical scavenging activity in rat brain (Yang et al., 2009). Oleracein E is also significantly explored for its neuroprotectant properties in the prevention and treatment of Parkinson’s disease (Sun et al., 2017).

5.15.5 Methyl 3, 4-dihydroxybenzoate

This compound an active for in-vitro antioxidants activity especially DPPH model (Azizuddin et al., 2010). It has been reported for the first time from B. procumbens. In another study, this compound used against three human cancer cell lines including MCF-7, NCI-H460, and SF-268, as well as human lymphocytic proliferation. This
compound also showed significant anti-inflammatory activity in a cell-based contemporary assay (Choudhary et al., 2009).

5.15.6 Eupalitin-3-0-β-D-galactopyanoside

This compound isolated for the first time from B. procumbens. Some studies reported that this compound showed promising immunosuppressive activity as well used as nephroprotective, inflammatory agents and autoimmune diseases (Pandey et al., 2005; Fathima & Parameswari, 2014). In another study, evaluated the inhibitory activity in bone resorption induced by parathyroid hormone and showed significant activity (Li et al., 1996).
6. STRUCTURE ELUCIDATION OF ISOLATED COMPOUNDS OF 
B. PROCUMBENS

6.1 Structure elucidation of Indole-3-carboxaldehyde

![Chemical structure (1)](image)

6.2 Physical properties:

- Physical state: white amorphous powder
- Molecular formula: C₉H₇NO
- Isolated quantity: 20 mg
- Melting Point: 193-198 °C
- Solubility: Soluble in methanol
- UV λ_max (MeOH): 206, 243, 255, and 298 nm
- EI-MS m/z (%): 146 [M+H]^+

6.3 Spectral Data

The ¹H NMR (DMSO-d₆) displayed resonances for the aromatic system at δ 8.14 (1H, br.d, J = 7.2 Hz; H-4), 7.28 (1H, br t, J = 7.2Hz; H-5), 7.22 (1H, br t, J = 7.2 Hz; H-6) and 7.46 (1H, br. d, J = 7.2 Hz; H-7). The ¹H-NMR spectrum also displayed resonances at δ 8.09 (1H, s; H-2), and 9.88 (1H, s; H-8). The ¹³C-NMR (BB and DEPT) displayed resonances for 9-carbons including six methine and three quartenary carbons. A methine signal at δ187.4 indicated the presence of aldehydic moiety in the molecule. The physical and spectral data were found identical to those reported in literature (Hiort, 2002). The spectra of this compound are in the Appendix 1.
6.4 Structure elucidation of 2-Hydroxybenoic acid (Salicylic acid)

Chemical structure (2)

6.5 Physical Properties

Physical state: white amorphous powder
Molecular formula: C₇H₆O₃
Isolated quantity: 10 mg
Melting Point: 154°C
FT-IR spectra: 3238 cm⁻¹ (OH), 1662 cm⁻¹ (C=O), 1613 cm⁻¹ (C=C), 759 cm⁻¹ (C=C-H)
Solubility: Soluble in methanol
EIMS m/z (%): 138 [M+H]⁺

6.6 Spectral data

The ¹H-NMR spectrums showed signals for the entire protons appeared in the aromatic region confirming the presence of a benzene ring. A doublet of doublet signals were observed at δ 7.82 (1H, J = 7.8, 1.5 Hz, H-6), while the proton at δ 7.53 (1H, J = 8.4, 1.5 Hz) exhibited triplet of a doublet was assigned to H-4. Two overlapped aromatic signals at δ 6.88 and 6.93 were assigned to H-3 and H-5, respectively. In the ¹³C-NMR spectrum of compound 2, one oxygenated carbon was observed at δ164.1(C-2) while one quaternary carbon was seen at δ 174.2 (C-7). ¹H- and ¹³C-spectral data for this compound were in accordance with Jadrijevi and Taka, (2004). (appendix 2).
6.7 Structure elucidation of 4-Hydroxybenzoic acid

![Chemical structure (3)](image)

6.8 Physical properties

Physical state: White crystalline solid
Molecular formula: C₇H₅O₃
Isolated quantity: 5 mg
Melting Point: 212-214 °C
FT-IR spectra: 3515 cm⁻¹(O-H), 3329 – 2722 cm⁻¹(carboxylic OH), 1706 cm⁻¹(C=O)
UV λ max (MeOH): 223 (3.85) nm, 311 (3.97) nm
EIMS m/z (%): 138 [M+H]⁺

6.9 Spectral Data

The ¹H-NMR spectrum of compound 3 depicted resonances at δ 7.94 (2H, d, J = 8.5 Hz, H-2, 6) and a carboxylic proton at δ 11.91(2H, br, s). The ¹³C-NMR spectrum displayed four methine and three quaternary carbons. The resonances at δ 177.03 and 160.32 were ascribed carbonyl carbon and oxygenated quaternary carbon of benzene ring, whereas signals at δ 131.52, 116.51 and 122.43 were due to aromatic methines and quaternary carbons. Comparison data (physical and spectral) with that reported in the literature (Rocha et al., 1994) identified compound 3 as 4-hydroxybenzoic acid. (Spectra of this compound are in appendix 3)
6.10 Structure elucidation of Oleracein E

![Chemical structure (4)](image)

6.11 Physical properties

Physical state: pale-white powder
Molecular formula: C_{12}H_{13}O_{3}N
Isolated quantity: 20 mg
Melting Point: 238-240 °C
UV $\lambda_{max}$ (MeOH): 318.5 nm (4.43), 290 nm (4.44).
FT-IR (KBr) $\nu_{max}$ (cm$^{-1}$): 3176, 2931, 1652, 1524, 1463, 1361, 1337, 1311, 12731068, 1021, 875.
Solubility: soluble in DMSO
EIMS $m/z$ (%): 218 [M+H]$^+$

6.12 Spectral Data

The $^1$H -NMR spectrum showed two aromatic signals at $\delta$ 6.48 s and 6.49 were assigned to H-7 and H-10. Two downfield signals at $\delta$ 8.77 and d 8.81 were due to hydroxyl protons of C-8 and C-10 hydroxyl group. One midfield proton at $\delta$ 4.56 (1H, t, $J = 8.0$ Hz) was assigned to the H-10b. Downfield shift of signals at $\delta$ 4.56, 3.95, 2.89 inferred the presence of nitrogen. $^1$H NMR (DMSO-$d_6$) ($\delta$ ppm): 1.56 m, 2.58 m, 2.21 m, 2.41 m, 3.96 m, 2.88 m, 2.58 (2H, m), 6.48 (1H, s), 8.81 s, 8.77 s, 6.49 (1H,s), 4.56 (1H, t, $J = 8.0$ Hz) (figure 11). $^{13}$C-NMR (DMSO-$d_6$) ($\delta$ ppm): 27.3 (C-1), 36.6 (C-5), 115.3 (C-7), 144.2 (C-9), 128.4(C-10a), 55.5 (C-10b). The data were found identical to those reported in literature (Xiang et al., 2005) (Appendix 4).
6.13 Structure elucidation of Methyl 3, 4-dihydroxybenzoate

\[
\begin{array}{c}
\text{Chemical structure (5)}
\end{array}
\]

6.14 Physical properties

Physical state: Brown amorphous powder
Molecular formula: C\textsubscript{8}H\textsubscript{8}O\textsubscript{4}
Isolated quantity: 12 mg
Melting Point: 134-138 °C
FT-IR (CHCl\textsubscript{3}) \textit{v}\textsubscript{max}: 3520 cm\textsuperscript{-1}(OH), 1718 cm\textsuperscript{-1}(C=O), 1608 cm\textsuperscript{-1} (C=C)
UV (MeOH) \textit{\lambda}\textsubscript{max}: 256 nm (3.74)
EIMS \textit{m}/\textit{z} (%):168 [M+H]\textsuperscript{+}

6.15 spectral data

The \textsuperscript{1}H NMR spectra of compound 5 showed the signals of 3H of the ABC system in aromatic ring by \(\delta\) 7.38 (brs, H-2), 7.32 (brd, \(J=8\)Hz, H-6) and 6.79 (d, \(J=8\) Hz, H-5) besides the signal at \(\delta\)H 3.69 (s, 3H, OCH\textsubscript{3}) (figure 13). The \textsuperscript{13}C NMR data confirmed this proposed benzoyl methyl ester structure by the signals at \(\delta\)C 166.5 (C=O of ester), 150.2, 145.0, 122.4 (C-4, C-3 and C-1, respectively), 122.0, 116.1, 115.0 (CH-6, 2 and 5) and 51.2 (OCH\textsubscript{3} of ester). Comparison data (physical and spectral) with that reported in the literature (Gerothanassis et al., 1998) identified compound 5 as methyl 3, 4-dihydroxy benzoate. (Appendix 5)
6.16 Structure elucidation of Eupalitin-3-\(\beta\)-D-galactopyranoside

![Chemical structure (6)](image)

6.17 Physical properties

Physical state: yellow amorphous solid
Molecular formula: \(C_{23}H_{24}O_{12}\)
Isolated quantity: 1200 mg
Melting Point: 93-98 °C
Solubility: soluble in DMSO
Optical density: \([\alpha]_D-26.6\) (c = 0.03, Methanol)
FT-IR spectra: (3400, 1600, 1584 and 1512 cm\(^{-1}\)).
UV (MeOH) \(\lambda_{\text{max}}\) nm: 270, 342 nm.
ESI-MS \(m/z\): 493[M+H]\(^+\)

6.18 Spectral Data

\(^1\)H-NMR spectrum showed peaks for aromatic protons at \(\delta\) 8.14 d (2H, \(J = 8.2\) Hz, H-2', H-6'), 6.85 d (2H, \(J = 8.2\) Hz, H-3', H-5'), 6.89 s (H-8), 10.19 s (H-OH/H-4'), 12.57 s (H-OH/H-5), 3.90 s (H\(_3\)-OCH\(_3\)/H-6), 3.72 s (H\(_3\)-OCH\(_3\)/H-6). \(^1\)H-NMR also displayed signals for sugar moiety at \(\delta\) 5.41d (1H, \(J = 7.6\) Hz, H-1''), and 3.54-4.82 m (6H, H-2'', 3'', 4'', 5'', 6''b, 6''b). Structure of the compound was further confirmed from 2D-NMR spectra (COSY, HSQC, and HMBC). All the physical and spectral data were unambiguously matched with previously reported compound Eupalitin-3-\(\beta\)-D-galactopyranoside (Li et al., 1996). The spectra of this compound available are in appendix 6.
7. SUMMARY

*Boerhavia procumbens* is an herbaceous, perennial creeping weed and native of Pakistan, belongs to the family Nyctaginaceae. *B. procumbens* has been used in the treatment of various ailments including asthma, cough and liver disorders (jaundice, hepatitis, etc.). In the present research work, methanolic extract, its various solvents soluble fractions and isolated compounds of sample plant were screened for various biological activities (*in-vitro* and *in-vivo*) along with chemical composition to provide a valid scientific rationale to its ethno-medicinal uses.

During these studies, aerial parts and roots of *B. procumbens* were subjected to chemical composition of proximate, mineral and amino acid. In the proximate analysis, the protein and inorganic matter of the roots were significantly (p<0.05) higher than the aerial parts. The highest value of Nitrogen-free extract was found in aerial parts as compared to roots. The inorganic matter was also analyzed for its mineral contents; the calcium and potassium were significantly (p<0.05) higher in concentration in the aerial parts than roots. Furthermore, the iron content was found higher in roots as compared to aerial parts. The essential amino acids, such as arginine and tryptophan was found significantly (p<0.05) higher in roots than aerial parts while non essential amino acid, namely, aspartic acid, glutamic acid and alanine were reported in higher concentration in aerial parts than roots of studied plant. Obtained results showed that *B. procumbens* was found as a rich source of nutrients & heavy metals. some toxic metals were found within the safer and permissible limits. So this plant could be used as a supplement for human consumption and can be added in fodder for livestock.

Qualitative phytochemical screening of sample plant showed that phenols, flavonoids and cardiac glycosides were present in crude methanolic extract while only phenolic and flavonoids contents were determined quantitatively in extract and its different solvent fractions. The phytochemical analysis proved that *B. Procumbens* was a rich source of plant secondary metabolites.

The crude extract and various fractions of studied plant were screened for antioxidant activity (DPPH). The EtOAc fraction (82.22 %) showed maximum DPPH scavenging activity followed by hexane fraction (64.78 %). The results obtained in the present
study indicate the sample plant as a potential source of natural antioxidants. The antibacterial activity of DCM and EtOAc fractions showed significant zone of inhibition followed by hexane fraction against *X. campestris* while other fractions showed mild antibacterial activity. In case of antifungal activity only hexane fraction showed comparable inhibition against *F. oxysporum* to that of standard while the crude extract, DCM and EtOAc fractions showed no activity against *A. Niger* and *A. Flavus*. In the present study the results of the antimicrobial activity encourages the use of BP for the treatment of most common microbial diseases. Furthermore, roots extract showed a very strong cytotoxicity against brine shrimps at high concentration (1000 µg/mL) while mild activity was observed at lower concentrations (100 and 10 µg/mL), respectively. The crude extract as well as different fractions were examined for their larvicidal effect against the 3rd instar larvae of *Culex quinquefasciatus* after 24 hrs of exposure. The leaf extract and hexane fraction showed highest percent mortality (100 % and 96 %) at high concentration (400 µg/mL). The Phytotoxic bioassay of crude extracts and fractions were tested against *Lemma minor* in order to evaluate its promoter or inhibitor effect on plant growth. The DCM fraction showed outstanding phytotoxic potential (50, 63, and 80 %) while hexane fraction showed mild activity (13, 20 and 26 %) at the test concentrations of 10, 100 and 1000 µg/mL, respectively.

The isolated compounds (1, 2, 4, 5 and 6) of *B. Procumbens* were evaluated for their leishmanicidal effect against promastigotes of *L. major*. Compound 5 and 4 showed a significant antileishmanial activity. The Oleracein E (4) and Eupalitin-3-O-D-galactopyranoside (6) compounds depicted good inhibition against acetylcholinesterase enzyme in comparison with standard drug, Galanthamine.

Five of the isolated compounds of *B. Procumbens* were also screened for Treg cell proliferation assay and compound 4 was found most active with percent inhibition of 83.51 % followed by compound 1 and 6 with percent inhibition of 32.02 and 24.18 % respectively, while PMA (Phorbol myristate acetate) was used as control. The cytotoxicity effect of the tested six compounds was found non cytotoxic. Except compound 4 which showed less cytotoxic effect against NIH 3T3 cell lines at the test concentration of 100 µM.
*B. Procumbens* extract was screened for its toxic effect and observed to have no toxic effect 24 hrs evaluation times. The extract was found very safe in acute toxicity test in mice. The crude extract of *B. Procumbens* showed significant effect in antinociceptive activity at dose of 400 mg/kg. Similarly, the anti-inflammatory activity of extract (200 & 400 mg/kg) was significant (*p*<0.05, *p*<0.01, respectively) after 3 hrs of carrageenan administration and this condition remained significant till 5 hours. The isolated compound (Eupalitin-3-O-β-D-galactopyranoside) of sample plant at a dose of 60 mg/kg exhibited significant effect against CCl₄ induced hepatotoxicity in rats with maximum reduction in the serum enzyme levels. The results of this study strongly indicated that studied plant had potent hepatoprotective action against CCl₄ induced hepatic damage in rats.

In phytochemical investigation, ethyl acetate and DCM fractions were subjected to various chromatographic techniques which led to the isolation of six pure compounds. Structures of the isolated compounds were elucidated using advanced spectroscopic and spectrometric techniques i.e. $^1$H-NMR, $^{13}$C-NMR, COSY, HMBC, NOESY, HSQC, IR, UV, EI-MS, and EIS-MS. All the six compounds were previously reported but first time isolated from this plant. Namely, Indole-3-carboxaldehyde (1), 2-Hydroxybenoic acid (Salicylic acid) (2), 4-Hydroxybenzoic acid (3), Oleracein E (4), Methyl-3, 4-dihydroxybenzoate (5) and Eupalitin-3-O-β-D-galactopyranoside (6).
8. CONCLUSIONS

1. In addition to the medicinal properties, the whole plant and various parts of *B. procumbens* was found as a rich source of nitrogen-free extract, protein, calcium, potassium, iron, arginine, tryptophan, aspartic acid and glutamic acid.

2. In the preliminary phytochemical screening, the crude extract and its subsequent solvent fractions revealed the presence of alkaloids, phenolic, flavonoids, saponins, tannins, and steroidal glycosides.

3. The *in-vitro* activities such as radical scavenging activity (DPPH), antibacterial, phytotoxic and larvicidal activities showed significant effect while cytotoxic (Brine shrimp) and antifungal activities were found moderate in crude extract and different solvent fractions of *B. Procumbens*.

4. The isolated compounds (4, 5 and 6) of sample plant showed significant antileishmanial activity and considerable inhibition of acetylcholineesterase enzyme while compound 4 was the most active in immune modulatory assay.

5. The *in-vivo* activities were carried out on crude extract and the results showed that *B. Procumbens* has potential antinociceptive and anti-inflammatory activities. The hepatoprotective activity (both *in-vitro* & *in-vivo*) was conducted on pure compound and showed protective effect against CCl₄ induced hepatotoxicity in rats.

6. In the present study, total six compounds were isolated from DCM and ethyl acetate fractions of *B. Procumbens*. These compounds are Indole-3-carboxaldehyde (1), 2-Hydroxybenoic acid (Salicylic acid) (2), 4-Hydroxybenzoic acid (3), Oleracein E (4), Methyl-3, 4-dihydroxybenzoate (5) and Eupalitin-3-O-β-D-galactopyranoside (6).
9. RECOMMENDATIONS

1. The *B. procumbens* is a rich source of various nutrients and some toxic metals of studied plant were found within the safer and permissible limits. Therefore this plant could be used as a herbal food supplement and can be added in fodder for livestock.

2. It is recommended that more studies are required on the extract of *B. Procumbens* to isolate and identify the active compound(s) responsible for its antioxidant, antimicrobial, Treg cell proliferation assay, leishmanicidal and cytotoxic properties.

3. Further *in-vivo* studies and clinical trials are needed to determine the efficacy, safety and mechanisms of action of isolated compounds prior to application in the pharmaceutical industry as natural therapeutic agents.

4. The real efficacy and identifying the unknown relative active principle in the crude extract of *B. Procumbens* are important for the advancement of effective and safe anti-inflammatory and analgesic therapy.

5. The Eupalitin-3-\(O-\beta-D\)-galactopyranoside compound showed significant effect against CCl\(_4\) induced liver toxicity but it needs further investigation in order to develop a new lead compound for the management of hepatotoxicity.
10. LITERATURES CITED


Waller (ed.). American Chemical Society Symposium Series 330, ACS, Washington, D.C.


Stewart, J. L. 1920. Nyctaginaceae in Punjab plants, comprising botanical and vernacular names and uses of most of the trees, shrubs, and herbs of economical value, growing within the province, government press, public works department. Lahore page:182.


Williams, J. T., and Z. Ahmad. 1999. A report on priorities for medicinal plants research and development in Pakistan, IDRC, MAPPA, New Delhi, India


APPENDICES

Appendix 1:

EI-MS spectra of compound 1.

Proton NMR spectra of compound 1
Appendix 2:

Proton NMR spectra of compound 2

EI-MS spectra of compound 2
Appendix 3:

Proton NMR spectra of compound 3.

EI-MS spectra of compound 3.
Appendix 4:

$^{1}$H NMR Spectra of Compound 4

$^{13}$C NMR spectra of compound 4
Appendix 5:

\[ \text{H NMR Spectra of compound 5} \]

\[ \text{C NMR Spectra of compound 5} \]
Appendix 6:

$^1$H NMR Spectra of compound 6.

$^{13}$C NMR spectra of compound 6.
Appendix 7: chemical structure of the isolated compounds from genus *Boerhavia*

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\text{OH} & \text{O} \\
\text{OH} & \text{O} \\
\end{align*}
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| 10 | \[
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\text{H} & \text{O} \\
\text{RO} & \text{O} \\
\text{OH} & \text{OH} \\
\end{align*}
\] \hspace{1cm} R = \text{CH}_3 |
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\text{RO} & \text{O} \\
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R_2 &= \text{H}
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47

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\text{HO}
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