

Phytochemical, Pharmacological and GC-MS Characterization of the Lipophilic Fraction of *Monothea buxifolia*

Shabnam Javed^{1*}, Amna Shoaib², Zaid Mahmood³, Shaista Nawaz⁴, Khalid Mohammed Khan⁵

¹Institute of Chemistry, University of the Punjab, Quaid-e-Azam Campus, Lahore-54590, Pakistan.

²Institute of Agricultural Sciences, University of the Punjab, Lahore, Pakistan

²Department of Chemistry, The University of Lahore, Pakistan.

³Food and Biotechnology Research Centre (FBRC), Pakistan Council of Scientific & Industrial Research Laboratories Complex, Lahore-54600, Pakistan.

⁴H. E. J. Research Institute of Chemistry, International Center for Chemical and Biological Sciences, University of Karachi, Karachi-75270, Pakistan.

Corresponding author: shabnam.chem@pu.edu.pk, 092-323-4830505

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Abstract

Monothea buxifolia [(Falc.) A. DC.] indigenous to northern Pakistan is a broad-leaved evergreen traditional plant of pharmacological versatility. The present investigation focused on evaluating the phytochemistry, nutritional profile, analgesic activity, cytotoxicity and GC-MS profile of *M. buxifolia*. The result showed aerial parts of *M. buxifolia* had high content of carbohydrate and protein, along with sufficient amounts of macro and micronutrients. The lipophilic *n*-hexane fraction of *M. buxifolia* exhibited the presence of saponins (29.30%), flavonoids (18%) and phenols (0.46 mg GAE/100 g). Analgesic activity of lipophilic fraction was equivalent to standard drug (Aspirin) as determined by the tail flick method. The F1 (oily liquid) of lipophilic fraction showed significant cytotoxicity against lung adenocarcinoma (A549) and liver (HepG2) cell lines). GC-MS profiling identified *cis*-vaccenic acid followed by *n*-hexadecanoic acid and hexadecanoic acid, methyl ester as major bioactive compounds in the lipophilic oily sub-fraction of *M. buxifolia*. These results support the use of oily sub-fraction of *M. buxifolia* as an effective and safe candidate for treatment of cancer cell lines.

Keywords: Gurgura, Macro micronutrient, Phytoconstitutents, Tail-flicking method, MTT assay

Introduction

Monothea buxifolia (Falc.) A. DC. is an economically and medically important plant, native to Pakistan, distributed in North-West regions of the country. It is used as folk medicine in different treatments such as a hematinic, laxative, digestive, anthelmintic, antipyretic, and urinary disorders, In Pakistan, local herbal medicine practitioners utilized its various parts against stomach ailments, cystitis, headache, toothache, diabetes, cough, jaundice, and dermatitis (Ullah et al., 2016). *M. buxifolia* has been investigated for hepatoprotective, anticancerous, antinociceptive, antipyretic, anti-inflammatory, cytotoxicity and urease enzyme inhibitory potentials due to high level of high antioxidant activity and diverse classes of flavonoids, phenolics and terpenoids derivatives (Burki et al., 2019; Ali et al., 2020).

Generally, phytochemicals exhibit fairly low bioavailability as they are handled by the body as xenobiotics therefore the presence in the body is ephemeral (Holst and Williamson, 2008). Therefore, bioavailability of dietary phytochemicals to target cells facilitate their bio-efficacy to protect our health (D'Archivio et al., 2010). Chemical structures and dietary intake forms are among the key features affecting the bioavailability of phytochemicals (Selby-Pham et al., 2017). Lipophilic drugs or “fat friendly” are among the non-ionized drugs which diffuse easily in the body by penetrating the cell membrane as compared to delayed drug permeation by ionized hydrophilic drugs (Gu et al., 2007). Lipophilicity property of the phytochemical is also considered as the most significant in influencing ligand–target binding interactions, solubility, ADME (absorption, distribution, metabolism, and elimination) properties, as well as *in vivo* toxicological outcomes, and, therefore, the overall quality of the drug candidate (Chung and Terry, 2015). Antioxidant, functional, nutritional and structural contributions of many lipophilic phytochemicals (e.g. sterols, carotenoids and tocopherols) have been known in the human body (Raikos and Ranawana, 2017). Lipophilic phytochemicals have been reported to reduce cardiovascular associated risk by lowering plasma cholesterol (Dinu et al., 2018). Due to pharmacological versatility of *M. buxifolia*, the current study was performed to analyze phytochemical profile of lipophilic fraction of *M. buxifolia*, and to assess its analgesic activity in addition to nutritional assessment of aerial plant parts. Besides, oily sub-fraction of lipophilic fraction was isolated and examined for cytotoxic activity against two human cancer cell lines (lung A549 and liver HepG2), and GC-MS analysis was performed to identify bioactive components of in oily sub-fraction.

Material and Methods

Proximate and mineral assays of *M. buxifolia*

The aerial parts (stem, flowers, leaves, and spines) of *M. buxifolia* (GC. Bot. Herb. 815) were analyzed for moisture content, protein, carbohydrate, fats and ash (AOAC, 1990). Concentration of heavy metals (Mg and Fe) were recorded through an atomic absorption spectrometer, while carbon, hydrogen, nitrogen and sulphur were assessed through a C/S determinator.

Extraction of lipophilic fraction

Dried aerial plant's parts (5.6 kg) were crushed by a mechanical grinder into a coarse powder followed by maceration using methanol (15 L). After 7 days, the solvent was removed under vacuum by rotary evaporator, and the gummy mass was diluted with half liter water further partitioned using *n*-hexane to yield lipophilic fraction (170 g).

Quantitative and qualitative phytochemical analyses of lipophilic fraction

Preliminary phytochemical evaluation for the detection of phytochemicals in lipophilic fraction were performed using standard methods (Sofowora, 1993; Harborne, 1998). The amount of phenolic content in the lipophilic fraction was measured by the Folin–Ciocalteu scheme (Zheng et al., 2001), while flavonoids were determined by the method of Boham et al. (1994). Saponins were quantified using methods described by Obadoni and Ochuko (2001).

Analgesic activity of lipophilic fraction

Lipophilic fraction was evaluated for analgesic activity in albino mice (male) using tail flick model (Williamson et al., 1997) and trial was carried out following the ethical principles for laboratory

animal's care (Zimmerman, 1983). Albino mice weighing between 200-250 g were randomly divided into two groups, with five animals in each group. The animals in group 1 (control) received standard drug, aspirin (300 mg/kg) and group 2 received lipophilic fraction (300 mg/kg). Both groups received treatment by oral route. Pain was induced by placing mice on the analgesiometer with the tail freely protruding out of the holder, and the tail was dipped from the terminal portion radiant heat source. Intensity of the current passing through the naked nichrome wire was 5 ampere. Tail-flick latency was measured at different time intervals till 4.5 hours after the drug administration.

Sub-fractionation of lipophilic fraction and cytotoxic activity assays

Oily sub fraction (F1: yellow viscous oily mass: 3.2 g) obtained through vacuum liquid chromatography and lipophilic sub-fraction were examined for cytotoxicity activity against A549 (lung adenocarcinoma) and HepG-2 (hepatocellular carcinoma) cell lines using MTT (3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide) (Mosmann *et al.*, 1983). About 1 ml of each cell suspension was added in 2×24 well plates after diluting cancer cells (1×10^3 cells/mL). Following the incubation period of 24 hours at 37 °C, the media was first replaced with 1 mL of lipophilic fraction and sub-fraction, and after another 24 hours, the fractions were replaced with 1 mL dye reagent. Absorbance of the samples were recorded at 570 nm through microplate ELISA reader.

GC-MS of the oily sub fraction

GC-MS analysis of oily sub fraction was performed on gas chromatograph (Shimadzu GC-9A) equipped with capillary column (SPB-5) maintained with flame ionization detector at 220 °C. Carrier gas (N₂: 1.0 mL/min) was adjusted at initial temperature at 50 °C for initial 5 min, followed by increase in temperature (5 °C/min) up to 235 °C and finally sustained for 5 min. A column (HP-5 with dimensions: 25 m \times 0.22 mm and 0.25 μ m df) was used to complete analysis of the fraction.

RESULTS AND DISCUSSION

Nature has bestowed potential in plants to synthesize diversity of chemical compounds of therapeutic properties, while significance of many medicinal plants still need to be explored. *M. buxifolia* is one of the economically and medically important plants, enriched with many bioactive compounds (Khan *et al.*, 2020). Hence, in the current investigation aerial parts of *M. buxifolia* were initially analyzed for phytochemicals and mineral contents, then lipophilic fraction was assessed for phytochemistry and analgesic activity, and finally sub-fractionation of lipophilic fraction was tested for cytotoxic activity.

Proximate and mineral analysis of aerial parts of *M. buxifolia*

Initially phytochemical analysis indicated that aerial parts of *M. buxifolia* contained 71.80% carbohydrates, 20.60% protein, 1.75% fats, 0.30% ash and 5.55% moisture content, respectively (Table 1). Previous findings recorded carbohydrates (63-74%), protein (0.85-4.37%), fats (4.07-7.70%), ash (6.01-17.67%) and moisture (10.27-15.50%) content in medicinal plants including *Monothea buxifolia* *Geranium wallichianum* and *Saxifraga flagillaris* (Begum *et al.*, 2018). High

content of carbohydrate of *M. buxifolia* could serve as main energy donor, since crude protein and fat contribute only in small portion, while sufficient protein content may be helpful in fulfilling intake of dietary protein (0.8 g/kg of body weight) as prescribed in health guidelines (EFSA, 2012). Fat content may indicate therapeutic advantage in terms of preserving insulin (Nagao and Yanagita, 2010). Macro and micronutrients contribute in the curative property of the plant as these elements participate in structural-functional qualities of the plant. Currently, magnesium (3.10 ppm) and iron (1.60 ppm) were detected in reasonable amounts, while carbon, hydrogen, nitrogen and sulphur were present as 46.82%, 6.51%, 2.32% and 0.38%, respectively (Table 1). Role of magnesium in bone formation, energy metabolism and enzymatic catalysis has been established (Ishimi, 2010), while essentiality of iron in haemoglobin synthesis, oxygen transport and boosting body immunity is known (Soyano et al., 1999). Sere and Vijayalakshmi (2018) also recorded high levels of magnesium and iron in leaves of medicinal plant, *Alternanthera sessilis*.

Table-1. Proximate and mineral analysis of aerial parts of *M. buxifolia*

Nutrients	%
Moisture	5.55
Ash	0.30
Fat	1.75
Protein	20.60
Carbohydrates	71.80
Elements	
Carbon (C)	46.82
Hydrogen (H)	6.51
Nitrogen (N)	2.31
Sulphur (S)	0.38%
	ppm
Magnesium Mg)	3.10
Iron (Fe)	1.60

Qualitative and qualitative phytochemical determination of lipophilic fraction

Lipophilic fraction showed presence of saponins, phenols, flavonoids, sterols and terpenoids (Table-2), while qualitative assays revealed 29.30% saponins, 0.46 mg GAE/100 g phenols and 8.0% flavonoids (Table-3). These results may indicate a large number of diverse bioactive compounds in the plant extracts, and the solvent utilized was also suitable to extract these bioactive compounds present in *M. buxifolia* (Ali et al., 2018). Jan et al. (2013) in their findings correlated the antioxidant activity of *M. buxifolia* with the phenolics and flavonoids contents of the fruit. Therefore, phenolic and flavonoid contents may be indication of potent immune-boosting agents due to their unique structural and functional properties. Furthermore, antioxidant, anticancer, and cardioprotective activity of the polyphenolics and flavonoids have been evidenced (Vlaisavljević et al., 2019).

Table-2. Qualitative phytochemical analysis of *M. buxifolia* lipophilic fraction.

Phytochemicals	(+) = present, (-) = absent, (++)= 50% or > 50 %
Alkaloids	-
Terpenoids	++
Sterols	++
Saponins	+
Phenols	+
Flavonoids	+
Tannins	-

Table-3. Total phenol, flavonoid and saponin content of *M. buxifolia* lipophilic fraction.

Phytochemicals	Total content
Phenol	0.46 (mg GAE/100 g)
Flavonoid	18 (%)
Saponins	29.3 (%)

Analgesic activity of *M. buxifolia* lipophilic fraction

Analgesic activity of lipophilic fraction of *M. buxifolia* was comparable with the standard drug Aspirin at same dose (300 mg/kg) at different time intervals (Table-4). In comparison to aspirin (standard drug), the lipophilic fraction exhibited significant analgesic activity in the tail flick method by increasing the latency time of responses. After two hours, the peak activity was observed in either treatment. Moreover, no death or signs of toxicity was observed with lipophilic fraction, which proposes that fraction and its dose were tolerable and safe for the mice. The long duration of analgesic activity of the lipophilic fraction of *M. buxifolia* proposed effectiveness of active metabolite in the fraction and these metabolites could also be accountable for activation of opioid receptors. The tail flick method is generally used to evaluate centrally acting analgesics, which may suggest that the lipophilic fraction activates the opioid receptors in the central nervous system (Hijazi et al., 2016).

Table-4. Analgesic effect of lipophilic fraction of *M. buxifolia* in tail response of mice.

Reaction Time (hour) after administration of sample	Lipophilic fraction (300 mg/kg)	Aspirin (300 mg/kg)
0 h	2.37 ^b ± 0.12	1.01 ^d ± 0.23
0.5 h	2.18 ^c ± 0.33	4.80 ^a ± 3.13
1 h	4.23 ^a ± 0.51	3.80 ^b ± 2.10
2.0 h	4.85 ^a ± 0.13	4.60 ^a ± 0.31
2.5 h	4.75 ^a ± 0.14	3.40 ^b ± 0.42
3 h	3.38 ^b ± 0.75	3.80 ^b ± 0.42
3.5 h	4.14 ^a ± 1.36	2.20 ^c ± 2.12
4.0 h	3.27 ^b ± 0.33	2.40 ^c ± 0.21
4.5 h	3.26 ^b ± 0.21	2.60 ^c ± 0.12

± indicate standard errors of mean of three replicates. Values with different letters show significant difference ($p \leq 0.05$) as determined by LSD-test.

Cytotoxic effect of lipophilic oily sub-fraction

The cytotoxic effect of lipophilic fraction and oily sub-fraction of *M. buxifolia* were evaluated based on effectiveness of fraction that give at least 50% of the cancer cell survivability (IC_{50}) (Table-5). The four categories of extracts have been proposed very active ($IC_{50} \leq 20 \mu\text{g/mL}$), moderately active ($IC_{50} > 20\text{--}100 \mu\text{g/mL}$), weakly active ($IC_{50} > 100\text{--}1000 \mu\text{g/mL}$) and inactive ($IC_{50} > 1000 \mu\text{g/mL}$) (Baharum et al., 2014). IC_{50} for pure compounds or drugs is taken potent, when value $< 4 \mu\text{g/mL}$ (Lee and Houghton, 2005). Cytotoxic analysis showed doxorubicin (standard control) exhibited high cytotoxic effect (IC_{50} values $\leq 10.6 \mu\text{g/mL}$) in both the cell lines. Lipophilic fraction of *M. buxifolia* was moderately active as it depicted IC_{50} values of 67.40 and 77.20 $\mu\text{g/mL}$ for A549 and HepG2 cancer cell lines, respectively. However, oily sub-fraction of lipophilic fraction was found to be highly active (IC_{50} : 28.20, 15.21 $\mu\text{g/mL}$ for A549 and HepG2, respectively) (Table-5). Javed et al. (2016) has also documented moderate cytotoxicity of *M. buxifolia* hexane fraction (IC_{50} : 59.20 $\mu\text{g/mL}$) against the human lung cancer cell line NCI-H460. Difference in cytotoxic response of cancer cell towards lipophilic fraction and its oily-sub fraction might be due to difference in the bioactive compounds. Hence, oily-sub fraction lipophilic fraction obtained by vacuum liquid chromatography, effectively inhibited cancer cell growth. Vacuum liquid chromatography may be advantageous compared with other methods of extracting active components from plant material due to low cost and simplicity (Mroczek et al., 2020).

Table-5. Cytotoxic activity (IC_{50} values) of lipophilic fraction and sub-fraction of *M. buxifolia*.

Treatments	IC_{50} values ($\mu\text{g/mL}$)	
	A549	HepG2
Lipophilic fraction	67.40 ± 3.02	77.2 ± 2.01
Lipophilic oily sub-fraction	28.20 ± 4.01	15.20 ± 4.01
Control (Without Drug)	> 100	> 100
Doxorubicin	9.70 ± 1.82	8.50 ± 3.21

\pm standard error of the mean three replicates.

GC-MS analysis of lipophilic oily sub-fraction

GC-MS analysis of active lipophilic oily sub-fraction indicated occurrence of 12 compounds. (Table-6). Cis-vaccenic acid (**8**) was identified as most frequently occurring compound followed by *n*-hexadecanoic acid (**3**) and hexadecanoic acid, methyl ester (**2**). Cis-vaccenic acid (mono-unsaturated fatty acid) is biosynthesized in humans by hepatic fatty acid elongase 5, and, it considerably reduced (23%) growth of HT-29 human colon cancer cells (Banni et al., 2001). Numerous further studies have confirmed the anti-inflammatory effects of mono-unsaturated fatty acids (Aimola et al., 2016). Aparna et al. (2012) recorded high anti-inflammatory action of *n*-hexadecanoic acid (saturated fatty acid) through reducing production of the inflammatory mediators. Likewise, hexadecanoic acid methyl esters are reported to have anti-inflammatory, cancer preventive, hepatoprotective, antiarthritic, and anti-coronary properties (Krishnamoorthy and Subramaniam, 2014). Other compounds were classified among less frequently occurring compounds, however, these compounds have been known as important with rest to pharmacology. The linoleic acid esters (**4**, **5**) act as antitumour, anti-arthritis agent and anti-inflammatory mediator (Elizabeth et al., 2014). Lupeol, lup-20(29)-en-3-ol, acetate, (3 α)- and 12-Oleanen-3-yl- acetate,

(3 α) (**10-12**) have been stated for cytotoxic, antitumor, antifu, antimalarial, antiviral activities and anti-inflammatory potential (Maragreth et al., 2009).

Table-6. Pharmacological components identified in the lipophilic oily fraction of *M. buxifolia* [GC-MS study].

S. N	R.T	Name	M. F	M. W	Peak area (%)
1	17.22	Pentanedioic acid, (2,4-di-t-butylphenyl) mono-ester	C ₁₉ H ₂₈ O ₄	320	0.42
2	25.07	Hexadecanoic acid, methyl ester	C ₁₇ H ₃₄ O ₂	270	6.61
3	26	<i>n</i> -Hexadecanoic acid	C ₁₆ H ₃₂ O ₂	256	24.84
4	26.95	9,12-Octadecadienoic acid (Z,Z)-, methyl ester	C ₁₉ H ₃₄ O ₂	294	3.48
5	27.03	9-Octadecenoic acid (Z)-, methyl ester	C ₁₉ H ₃₆ O ₂	296	3.07
6	27.07	9-Octadecenoic acid, methyl ester, (E)-	C ₁₉ H ₃₆ O ₂	296	3.63
7	27.3	Methyl stearate	C ₁₉ H ₃₈ O ₂	298	2.25
8	28.07	cis-vaccenic acid	C ₁₈ H ₃₄ O ₂	282	40.28
9	28.13	Octadecanoic acid	C ₁₈ H ₃₆ O ₂	284	5.72
10	47.25	Lupeol	C ₃₀ H ₅₀ O	426	3.34
11	48.28	12-Oleanen-3-yl acetate, (3 α)-	C ₃₂ H ₅₂ O ₂	468	2.46
12	49.87	lup-20(29)-en-3-ol,acetate,(3 α)-	C ₃₂ H ₅₂ O ₂	468	3.90

Conclusion

In conclusion, the contemporary study specifies that aerial parts of *M. buxifolia* contained high content of carbohydrate and protein, and sufficient amount of macro and micronutrients. Lipophilic fraction contained saponins, phenols, flavonoids, sterols and terpenoids, while qualitative assays revealed substantial quantity of saponin, phenolics and flavonoids. Analgesic activity of lipophilic fraction was comparable to aspirin. The cytotoxic effect of lipophilic oily sub-fraction was better than lipophilic fraction against cancer cells. GC-MS profiling identified cis-vaccenic acid followed by *n*-hexadecanoic acid and hexadecanoic acid, methyl ester as major compound bioactive compounds in the lipophilic oily sub-fraction of *M. buxifolia*. These results support the use of oily sub-fraction of *M. buxifolia* as effective and safe candidate for treatment of cancer cell lines.

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Disclaimer

None

Conflict of Interest

The authors declare that they have no conflict of interest.

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Author's contribution

Shabnam Javed: Performed experiments, collected data, analyzed data and wrote first draft of the manuscript; **Amna Shoaib:** Wrote manuscript; **Zaid Mehmood:** Designed experiment; **Shaista Nawaz:** Performed Analgesic and cytotoxic activity assays; **Khalid Mohammed Khan:** Contributed in supervising chromatographic isolation and characterization of compounds

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