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Antioxidant, analgesic and anti-inflammatory activities of *in vitro* and field-grown Iceberg lettuce extracts

[Actividades antioxidante, antiinflamatoria y analgésica de extractos de lechuga Iceberg cultivada *in vitro* y en el campo]

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Abstract

Context: Iceberg lettuce is a nutrient rich edible crop containing vitamin B6, thiamin and folate. The increasing demand of natural products is moving the focus towards *in vitro* plant sources as factories for producing important secondary metabolites.

Aims: To evaluate the activities of *in vitro* propagated callus culture and field-grown of Iceberg lettuce, which can act as an alternate source of bioactive secondary metabolites.

Methods: The extracts were made with methanol, n-hexane, ethyl acetate and water for field-grown as well as *in vitro* cultured Iceberg lettuce plants and evaluated for their medicinal potential both *in vitro* and *in vivo* in rats**.**

Results: Total flavonoid and phenolic contents of plant extracts showed strong positive correlation with their antioxidant activities. The highest contents of flavonoids and phenolics were present in the methanolic extract of field-grown leaves. The antioxidant, anti-inflammatory and analgesic activities of examined samples in rats were observed as field-grown leaves extracts > callus extracts > regenerated shoots extracts. However, all of these activities in the extracts of callus culture were comparable to that of field-grown plant extracts.

Conclusions: This study showed that field-grown plants are comparably rich source of secondary metabolites, which showed multiple pharmacological activities in a significant way.

Keywords: analgesic; anti-inflammatory; antioxidant; callus; Iceberg lettuce; *Lactuca sativa*.

Resumen

Contexto: La lechuga iceberg es un cultivo comestible rico en nutrientes que contiene vitamina B6, tiamina y ácido fólico. La creciente demanda de productos naturales está moviendo el foco hacia fuentes de plantas *in vitro* como fábricas para producir importantes metabolitos secundarios.

Objetivos: Evaluar las actividades del cultivo de callos propagados *in vitro* y en el campo de la lechuga iceberg, que pueden actuar como una fuente alternativa de metabolitos secundarios bioactivos.

Métodos: Los extractos se hicieron con metanol, n-hexano, acetato de etilo y agua para plantas de lechuga Iceberg cultivadas *in vitro* y en el campo y se evaluaron para determinar su potencial medicinal tanto *in vitro* como *in vivo* en ratas.

Resultados: El contenido total de flavonoides y fenoles de los extractos de plantas mostró una fuerte correlación positiva con sus actividades antioxidantes. Los contenidos más altos de flavonoides y fenoles se encontraron en el extracto metanólico de hojas cultivadas en el campo. Las actividades antioxidantes, analgésicas y antiinflamatorias de las muestras examinadas en ratas se observaron como extractos de hojas cultivadas en el campo > extractos de callos > extractos de brotes regenerados. Sin embargo, todas estas actividades en los extractos de cultivos de callos fueron comparables a las de los extractos de plantas cultivadas en el campo.

Conclusiones: Este estudio demostró que las plantas cultivadas en el campo son una fuente comparable de metabolitos secundarios que muestra múltiples actividades de manera significativa.

Palabras Clave: analgésico; antiinflamatorio; antioxidante; callo; lechuga iceberg; *Lactuca sativa*.

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INTRODUCTION

Lactuca sativa L. is a major leafy vegetable of *Asteraceae* family (De Vries, 1997), which is mainly consumed as a fresh vegetable in salads. However, in some countries, it is either cooked, dried, pickled, or used as sauce. The cultivated *L. sativa* is widely known for its rich vitamin and mineral contents (Burtin, 2003). This crop is widely grown as home vegetable and also cultivated for commercial purposes all over the world. China, U.S.A., Spain, Italy, Japan and India are considered as the leading lettuce producing countries across the world (Mou, 2008). Iceberg lettuce is one of the most commonly consumed lettuce. Due to its moist crispiness, it is favorite over most varieties of lettuce. Additionally, it has low cholesterol and saturated fats and is a key source of vitamins (such as A, B6, C, K) and iron (Hu, 2003). It also contains a high content of dietary fibers and traces of omega fatty acids, which are beneficial for health. Another benefit that Iceberg lettuce offers is the presence of folate in it, which aids to combat heart disease (Johansson et al., 2007).

Lettuce has been used as folk medicine due to its medicinal importance. Lettuce holds numerous medicinal qualities including, anxiolytic, antioxidant, anticonvulsant, analgesic, antidepressant, anti-inflammatory and anticoagulant activities (Chu et al., 2002; Sayyah et al., 2004; Harsha and Anilakumar, 2013; Ismail and Mirza, 2015). According to traditional knowledge, lettuce can be used for treatment of anxiety, dry cough, insomnia, pain, rheumatic arthritis and neurosis (Berg et al., 2003). Leaves of lettuce are dynamic source of vitamin A, C and lactucopicrin, which has been tested to inhibit cancers (Chu et al., 2002). The beneficial effects of lettuce in avoiding cardiovascular disorders have also been reported in rats and humans (Nicolle et al., 2004). Lettuce is used to boost digestion and appetite and for the treatment of stomach problems (Sayyah et al., 2004). In all types of lettuce, a mild opiate-like substance called lactucarium is present that can assist to induce sleep and relaxation. Lactucarium is also used as hypnotic drug in asthma and bronchitis (Mohammad, 2013). The extracts of lettuce are used to make lotions and creams for the cure of burns and skin ailment. The vegetable is also thought to be associated with improvement of liver conditions (Mohammad, 2013).

Plants are well known as a valued source of a large number of important secondary metabolites such as agrochemicals, pharmaceuticals and food additives (Mulabagal and Tsai, 2004; Waheed et al., 2015; Manouze et al., 2017). More than 80% of about 30,000 known natural products are derived from plant sources. Plant cell and tissue culture is an attractive approach for the production of secondary products at an industrial level. The growing demand for renewable and natural products has transferred the focus on *in vitro* plant materials as possible factories for producing high-value secondary metabolites. Plant tissue culture provides a valuable alternative for plant micropropagation as well as for the production of bioactive substances under well-controlled conditions, irrespective of season. Cultured plant cells and tissues have the ability to synthesize and accumulate various medicinally important secondary metabolites. Different approaches are used to enhance yields of secondary products by *in vitro* production (Rao and Ravishankar, 2002). In most of the reports about medicinal plants, crude extracts derived from the field-grown plants are used for the assays. However, plant material obtained via tissue culture can also be used for animal studies to compare the effects. In the present study, field-grown and *in vitro* lettuce cultures, including callus and regenerated shoots, were examined for biological activities in rat models. The overall objective was to perform the comparative analysis of biological activities in both *in vivo* grown plants (field-grown) and *in vitro* derived tissues (callus and regenerated shoots) of Iceberg lettuce, by conducting different *in vitro* and *in vivo* assays.

MATERIAL AND METHODS

Chemicals

All the chemicals including ethanol, mercuric chloride, Murashige and Skoog medium, benzyl-

aminopurine, naphthalene acetic acid, n-hexane, ethyl acetate, methanol, gallic acid, quercetin, ascorbic acid, aluminum chloride, potassium ferricyanide, DPPH, diclofenac potassium, acetylsalicylic acid, carrageenan and Folin-Ciocalteu reagent were purchased from Sigma-Aldrich, USA.

Plant material

The leaves of *Lactuca sativa* var. *Capitata* '*Iceberg*' were obtained from a supermarket in Pakistan. The plant was identified by Dr. Muhammad Zafar, Department of Plant Sciences, Quaid-i-Azam University, Islamabad, Pakistan and voucher specimen (128085-B) was deposited in the Herbarium of Pakistan. This plant material, designated as 'field-grown', was simply washed, dried and then used for preparing extracts that were later used for bioassays.

Callus and shoots induction

Some of the obtained leaves were sterilized by dipping in 70% ethanol for 1 minute and then in 0.2% mercuric chloride solution for 20 seconds and finally rinsed 3 times with distilled water. The leaves were cut into 3 × 2 mm explants and were placed on Murashige and Skoog (MS) medium (Murashige and Skoog, 1962). When explants initiated regeneration, MS medium was supplemented with 2 mg/L benzylaminopurine (BAP) and 1 mg/L naphthalene acetic acid (NAA) for callus induction and 0.5 mg/L BAP and 0.1 mg/L NAA for shoots induction. Leaf explants were placed in a growth room at 25 ± 2°C under 16/8 hours light/dark cycle and 50–60% relative humidity (Dilshad et al., 2016a). Sixteen weeks old callus and regenerated shoots were used for biological analysis. This plant material was designated as *in vitro* propagated material.

Extraction and yield

The field-grown leaves and *in vitro* propagated plant material including callus and regenerated shoots were air dried and then crushed into powder by using Kitchen Grinder (GN-2837, National, Japan). The fine powder (100 g) was sequentially extracted by maceration in n-hexane (200 mL) for 5-days and was filtered by Whatman #1 filter paper (Sigma, USA). The residue was further sequentially extracted with ethyl acetate, methanol, and distilled water accordingly, each in 200 mL. The extracts obtained from each solvent were dried at 45°C under vacuum hood and stored at 4°C till further use.

Phytochemical analysis

Total phenolic content (TPC) was determined by Folin-Ciocalteu method while total flavonoid content (TFC) in plant extracts was determined by using an aluminum chloride colorimetric method as reported earlier (Ismail et al., 2017). The TPC was determined as mg of gallic acid equivalent (GAE) while TFC of the extracts was determined as mg of quercetin equivalent (QE) per gram of dry extract. The standard curve equations for gallic acid ($y = 0.0719x + 0.3563$) and quercetin ($y = 0.01x + 0.2312$) and the R^2 values (0.999, 0.996, respectively) were obtained.

Total reducing power and total antioxidant capacity

Total reducing power of extracts was determined by potassium ferricyanide (Dilshad et al., 2016b). Meanwhile, total antioxidant capabilities of the extracts were measured by phosphomolybdenum method (Ismail et al., 2016). Experiment was performed in triplicate at 1 mg/mL of extract. In both assays, DMSO was used as blank and results were presented as ascorbic acid equivalents (AAE). The standard curve equations for ascorbic acid ($y = 0.0298x + 0.8921$) and the R^2 value (0.9945) were calculated.

DPPH assay

Free radical scavenging activity of extracts was evaluated by measuring the discoloration of purple colored DPPH solution (Ismail and Mirza, 2015). A volume of 180 µL of DPPH solution (0.1 g/L) was added in each well (96-well plate), followed by addition of 20 µL of three different dilutions (250, 500, 750 and 1000 µg/mL) of each extract. The reaction mixture was mixed, and plate was incubated at 37°C in dark for 1 hour. The absorbance was recorded by using Elx-800 microplate reader (BioTek, USA) at wavelength of 517

nm. The assay was repeated in triplicates and ascorbic acid was used as positive control. For negative control, reagent solution without sample was used. Percentage free radical scavenging potential of extracts was derived from given formula [1]. The inhibitory concentration at which test sample showed 50% scavenging (IC_{50}) was determined by using Graph Pad Prism 5 software.

$$\% \text{ scavenging} = \left[\frac{\text{Absorbance of negative control} - \text{Absorbance of extract}}{\text{Absorbance of negative control}}\right] \times 100 \quad [1]$$

Animal studies

Based on the phytochemical analysis and antioxidant activities only methanol extracts were selected for animal studies. For each experiment adult male albino rats weighing about 160–180 g were selected. Rats were maintained at 25 ± 2°C with a 12 h light/dark cycle and were fed with water *ad libitum* and standard diet. The study design was approved by the Institutional Ethics and Biosafety Committee (approval letter code BCH-0278). All the precautions were carried out to minimize animal sufferings. Rats were divided into eleven groups containing seven rats in each group. Samples were administered orally to all groups. Group 1 was negative control and administered with saline (0.9%) only. Group 2 was positive control in which rats received 2.5 mg/kg, 5 mg/kg and 10 mg/kg body weight (b.w.) of standard drugs (acetylsalicylic acid for analgesic and diclofenac potassium for anti-inflammatory assays). Groups 3-5, 6-8 and 9-11 were given 50, 100 and 200 mg/kg dose of methanolic extract of field-grown leaves, callus and regenerated shoots, respectively.

Acute toxicity test

In this study was selected 500 mg/kg b.w. for oral toxicity due to the limitation of tissue culture extract. Acute toxicity of each extract was tested in rats at this dose by oral administration on seven rats per group as per OECD guidelines (Guideline, 2001). The rats were monitored for any behavioral changes or mortality at regular intervals for 24 h. The animals were further observed for next 7 days for any symptoms of delayed toxicity/mortality.

Hot plate analgesic assay

Hot plate method was performed to evaluate the analgesic effect of Iceberg lettuce extracts. In this method, pain was induced by heat using hot plate (IITC Life Science, USA) adjusted at 50 ± 2°C and its response was observed by measuring the latency time (i.e. time of first paw licking or jumping). Prior to drug treatment, the initial latency time (T_i) was noted by placing the rats on hot plate. After drug administration, the final latency time (T_f) was recorded for each group at the intervals of 0.5, 1 and 2 h, with a cut off time of 25 s (Eddy and Leimbach, 1952). Percentage analgesia was determined by using the given formula [2] and the results were compared with saline and acetylsalicylic acid.

$$\% \text{ analgesia} = \left[\frac{Tf - Ti}{Ti}\right] \times 100 \quad [2]$$

Anti-inflammatory assay

Carrageenan-induced inflammation test was performed to estimate the anti-inflammatory effect of extracts (Sajid et al., 2017). In this assay, saline was employed as a negative control while standard drug diclofenac potassium served as a positive control to compare the anti-inflammatory effects. To induce the paw edema, 100 µL of carrageenan (1% in saline) was injected into the left hind paw of each rat after one hour of dosage. Before the carrageenan induction, the paw volume was measured by using digital plethysmometer (UGO Basile, Italy), which served as the initial paw volume and then after the injection readings were taken at 1st, 2nd, 3rd and 4th hour. The percentage inhibition of edema was then calculated based on the formula [3, 4] as follows:

$$\text{Edema volume} = \text{respective hour paw volume} - \text{initial paw volume} \quad [3]$$

$$\% \text{ edema inhibition} = \left[\frac{\text{edema volume of control} - \text{edema volume of extract}}{\text{edema volume of control}}\right] \times 100 \quad [4]$$

Statistical analysis

Data was presented as mean along with standard deviation. Prism 7 software was used to find the correlation and data were analyzed by using ANOVA followed by Turkey multiple comparison

test. The results were considered to be significant at $p<0.05$.

RESULTS AND DISCUSSION

Total phenolic and flavonoid contents

Preliminary quantification of phytochemicals provides an evidence for the pharmacological potential of the plant. Being plant secondary metabolites, the phenolics having antioxidant activities play a key role in fighting oxidative stress, cell decease and cytotoxicity (Sen et al., 2010; Sajid et al., 2016). TPC of the tested extracts varied from 9.93 ± 1.12 mg GAE/g for the hexane extract of regenerated shoots to 82.41 ± 1.06 mg GAE/g for the methanolic extract of field-grown leaves (Table 1). On the other hand, TFC appeared in the range from 4.60 ± 1.25 to 48.83 ± 0.70 QE/g (Table 1). When comparing the solvent system, the methanolic extract demonstrated the highest total content of phenolics, whereas the lowest content was observed with hexane extracts. The overall order of TFC in extracts was found to decrease as: methanol > ethyl acetate > water > hexane. It has also been shown previously that the extraction of phenolics from the plant samples are influenced by the nature of solvent (Akowuah et al., 2005; Turkmen et al., 2006). In the present report, filed grown plants showed more TPC and TFC, strengthening previous findings, which demonstrated that conventionally propagated field-grown plants show higher extents of flavonoid and phenolic components as compared to the *in vitro* propagated plants (Parsaeimehr et al., 2010; Khorasani Esmaeili et al., 2015). When comparing the *in vitro* derived tissues, our results showed that callus had higher phenolic and total flavonoid contents than regenerated shoots. Physiological state of tissues can have impact on secondary metabolites levels that can be influenced by age of the tissues and culture medium composition (Iqbal and Srivastava, 2000).

Table 1. Total phenolic and total flavonoid contents of field-grown and *in vitro* grown plants of lettuce Iceberg extracts.

Extract	Total phenolic content (mg GAE/g DE)	Total flavonoid content (mg QE/g DE)
Field-grown leaves		
Hexane	15.86 ± 0.62**	9.80 ± 1.75*
Ethyl acetate	43.19 ± 0.98**	17.63 ± 1.12*
Methanol	82.41 ± 1.06*	48.83 ± 0.70**
Water	28.93 ± 1.48*	13.33 ± 1.76*
Regenerated callus		
Hexane	11.17 ± 1.23*	5.30 ± 1.05*
Ethyl acetate	39.60 ± 1.42*	12.53 ± 0.83**
Methanol	72.00 ± 0.66**	35.58 ± 1.61*
Water	24.23 ± 1.72*	10.00 ± 0.98**
Regenerated shoots		
Hexane	9.93 ± 1.12*	4.60 ± 1.25*
Ethyl acetate	38.00 ± 0.80**	7.89 ± 0.71**
Methanol	63.03 ± 0.96**	26.11 ± 1.74
Water	22.87 ± 1.27*	5.84 ± 0.92**

Values are expressed as mean (n=3) ± SD with * $p<0.05$, ** $p<0.01$ statistical significance as compared with control group. GAE: gallic acid equivalent; QE: quercetin equivalent; DE: dry extract.

Table 2. Total reducing power, total antioxidant capacity and DPPH inhibition of field-grown and *in vitro* grown plants of lettuce Iceberg extracts.

Plant extract	Field-grown leaves	Regenerated callus	Regenerated shoots
Total reducing power (mg AAE/g DE)			
Hexane extract	23.83 ± 1.10*	22.13 ± 2.60*	19.50 ± 1.20*
Ethyl acetate extract	60.37 ± 2.05*	51.53 ± 0.95**	39.37 ± 1.05*
Methanol extract	74.17 ± 0.91**	67.16 ± 0.85**	58.54 ± 1.19*
Water extract	51.77 ± 1.60*	47.83 ± 1.48*	35.73 ± 1.53*
Total antioxidant capacity (mg AAE/g of DE)			
Hexane extract	14.33 ± 0.65**	13.89 ± 1.04*	11.24 ± 0.70**
Ethyl acetate extract	25.80 ± 1.67*	20.85 ± 2.39*	18.85 ± 1.07*
Methanol extract	43.23 ± 0.75**	37.77 ± 1.17*	31.74 ± 2.14*
Water extract	27.30 ± 1.25*	23.35 ± 0.66**	21.13 ± 2.11*
IC_{50} of DPPH scavenging assay (mg/mL)			
Hexane extract	1.65 ± 0.06	1.57 ± 0.06	2.44 ± 0.08
Ethyl acetate extract	0.66 ± 0.05*	0.74 ± 0.04*	0.80 ± 0.04*
Methanol extract	0.31 ± 0.03**	0.33 ± 0.01**	0.37 ± 0.02**
Water extract	1.23 ± 0.02**	1.39 ± 0.03**	1.36 ± 0.04*
Ascorbic acid	0.02 ± 0.001***	0.02 ± 0.001*	0.02 ± 0.001***

Values are expressed as mean (n=3) ± SD with *p<0.05, **p<0.01, ***p<0.001 statistical significance as compared with control group (reagent solution without sample). AAE: ascorbic acid equivalent; DE: dry extract.

Total reducing power

Total reducing power (TRP) of the extract reflects the antioxidant activity, which is due to the ability of reductants to donate hydrogen to the free radicals. In TRP, the incidence of reductants (antioxidants) in the sample causes the reduction of the Fe^{3+} in ferricyanide complex to the Fe^{2+} form and resulting in transformation of yellow color of the solution to various hues of blue and green, depending upon the extent of the reducing power of the samples (Jafri et al., 2017). The reducing power of plant extracts was expressed as mg of ascorbic acid equivalent per gram dry extract as shown in Table 2. Among the examined extracts, the highest reducing power activity was obtained in methanolic extract of *in vivo* leaves as 74.17 ± 0.91 mg AAE/g. The overall sequence for reducing power was as follows: *in vivo* leaves > callus > regenerated shoots. In both (field-grown and *in vitro*), the methanol extract illustrated the highest reducing power activity followed by other extracts in decreasing order as: ethyl acetate > water > hexane. Antioxidants such as flavonoids and phenolics in the extracts of Iceberg lettuce may contribute to their reducing power as reports suggest that phenolics and flavonoids have been associated with antioxidative potential in biological systems (Kim et al., 2006).

Total antioxidant capacity

Total antioxidant capacity (TAC) of the plant extracts was quantitatively evaluated by using phosphomolybdenum assay. This assay works on the principle of reduction of Mo(VI) to Mo(V) by the antioxidants present in the sample and subsequent formation of green color phosphomolybdenum (V) complex at acidic pH, which is measured spectrophotometrically (Prieto et al., 1999). TAC was determined as AAE/g of dry extract and was found to be in the sequence: field-grown leaves > callus > regenerated shoots (Table 2). The results exemplified that the highest antioxidant

activity (27.20 ± 1.26 mg AAE/g) was shown by the methanolic extract of field-grown leaves, whilst the lowest (11.24 ± 0.70 mg AAE/g) was exhibited by n-hexane extract of *in vitro* grown regenerated shoots. Some previous reports suggest that polyphenols and flavonoids significantly contribute to the TAC of plants and thus provide protection against oxidative damage (Sharififar et al., 2009; Khan et al., 2012). Levels of different nutrients and certain other factors such as light can influence the secondary metabolite production (Miehe-Steier et al., 2015). It could be that field-grown leaves showed more total antioxidant capacity due to availability of more nutrients in soil compared to *in vitro* regenerated plants.

DPPH inhibition

DPPH assay is economical and short-time method to evaluate the antioxidant ability of plant samples (Khan et al., 2012). All the extracts showed scavenging activities against DPPH free radicals in a dose-dependent manner (250–1000 µg/mL). The results depicted that methanol extracts of all plant samples showed the highest activity against DPPH radical followed by water, ethyl acetate and n-hexane extracts (Fig. 1, Table 2). At 1000 µg/mL concentration, methanol extracts of field-grown leaves, callus and regenerated shoots demonstrated 88.27, 83.60 and 80.43% DPPH inhibition, respectively (Fig. 1). The calculated IC_{50} values showed that field-grown leaves (IC_{50} = 306.7 mg/mL) exhibited the highest activity as compared with regenerated callus (IC_{50} = 331.4 mg/mL) and shoots (IC_{50} = 367.6 mg/mL) as presented in Table 2. Our findings are in accordance with Singh et al. (2014) who reported that methanolic extract showed higher antioxidant activity than extracts of other solvents such as water, ethyl acetate and n-hexane. Scavenging results in this assay are consistent with results of TPC and TFC, which reinforce the idea that hydroxyl group present in phenolic compounds may have imparted scavenging potential. In addition, presence of flavonoids, the most renowned class of phenolics, also perform their action by scavenging or chelation (Yildirim et al., 2000).

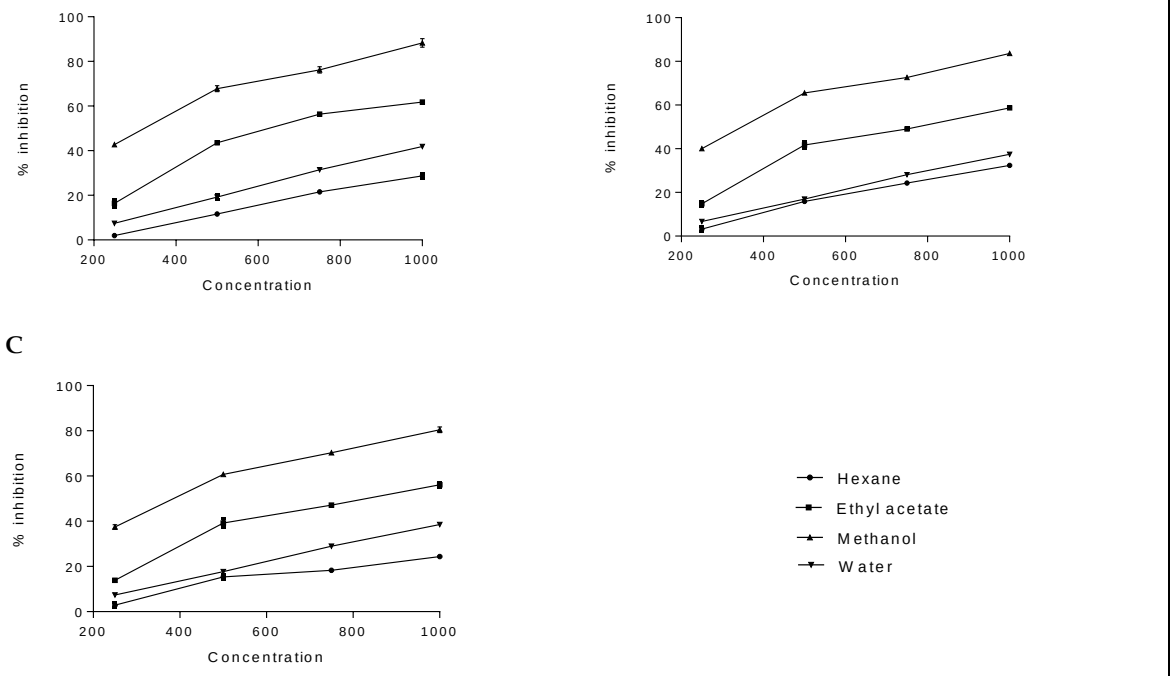

Figure 1. DPPH radical scavenging activity of different extracts of lettuce Iceberg by different solvent at different concentration (µg/mL).

(A) field-grown leaves; **(B)** regenerated callus; and **(C)** regenerated shoots. Each value is represented as mean ± SD (n=3).

Animal studies

Acute toxicity test

No mortality was observed for any extract at 500 mg/kg (p.o). None of the extracts produced notable changes in behavior during the time of observation.

Analgesic activity

In this study, central analgesic activity including spinal reflexes was investigated by thermal nociception model. For this purpose, hot plate assay was performed, which is considered as an easy and suitable method. The results indicated that among tested extracts, field-grown leaf extracts showed the highest analgesic potential (Fig. 2A). The activity was determined in time-dependent manner and highest activity was noted at 1 h after the sample induction. The highest activity was calculated for field-grown leaves (75.74%) followed by regenerated callus (66.52%) and regenerated shoots (59.40%), respectively at 200 mg/kg. Overall results exhibited concentration-dependent activity. Acetylsalicylic acid showed IC_{50} = 0.8 mg/mL while field-grown, callus and regenerated shoot extracts delivered IC_{50} values = 34.45, 65.02, and 103.8 mg/mL, respectively. Previously, the presence of flavonoids was described in some species of *Lactuca* and these flavonoids are considered to have a role in inhibiting the key enzyme prostaglandin synthetase (Hugar et al., 2010; Ismail and Mirza, 2015). As prostaglandins are involved in the process of pain perception, thus it can be proposed that reduced availability of prostaglandins due to the presence of flavonoids might be responsible for analgesic activity (Kayani et al., 2016).

Anti-inflammatory activity

Carrageenan is extensively used to induce hind paw edema in rodents to investigate the possible anti-inflammatory effect of herbs or drugs. This *in vivo* investigation method is actually a biphasic model. The early phase (1-2 h) of the inflammation contributes to release of serotonin, bradykinin, histamine and other similar substances, which later on causes the increased synthesis of prostaglandins from the nearby region of injured tissues. The final phase of 3-4 h is characterized by the peak volume of hind limb. During this stage, edema attains its highest volume due to the elevated level of kinin-like substances such as prostaglandins, lysosome and proteases (Araruna and Carlos, 2010). The anti-inflammatory response exhibited by the field-grown leaves extract (76.90%) was greater than that of the *in vitro* callus (70.86%) and regenerated shoots (60.76%) extracts (Fig. 2B). However, it was observed that the activity was comparable in field-grown leaves extract and callus extract. As far as the IC_{50} value is concerned, diclofenac potassium exhibited 8.4 mg/mL while the field-grown leaves, callus and shoots demonstrated 37.6, 80.4 and 104.6 mg/mL, respectively. The present study demonstrated that oral dose of methanol extracts inhibited the edema formation primarily from the first hour and throughout all stages of inflammation. Our findings are in consensus with the previous results of Ismail and Mirza (2015), who reported *L. sativa* cultivar Grand Rapids as an analgesic and anti-inflammatory agent. The phytochemical studies of *L. sativa* have revealed the presence of simple phenolics, saponins and triterpenoids that have been linked with anti-inflammatory properties (Suh et al., 1998; Hefnawy and Ramadan, 2013). Hence, it might be possible that anti-inflammatory and analgesic activities of *L. sativa* are related to saponins and triterpenoids content present in it.

Correlation between phytochemicals and plant activities

To determine the phytochemical linkage with the activities of plant extracts, a correlation was developed using Graph Pad Prism 7 software (Table 3). In case of field-grown leaves, it was observed that all of the antioxidant assays and animal studies showed strong positive ($p<0.05$) correlation with total flavonoid and phenolic contents. Correlation statistics of regenerated callus exhibited that all the activities showed positive correlation except total antioxidant capacity. In this case, both total flavonoid and phenolic content had p values greater than 0.05.

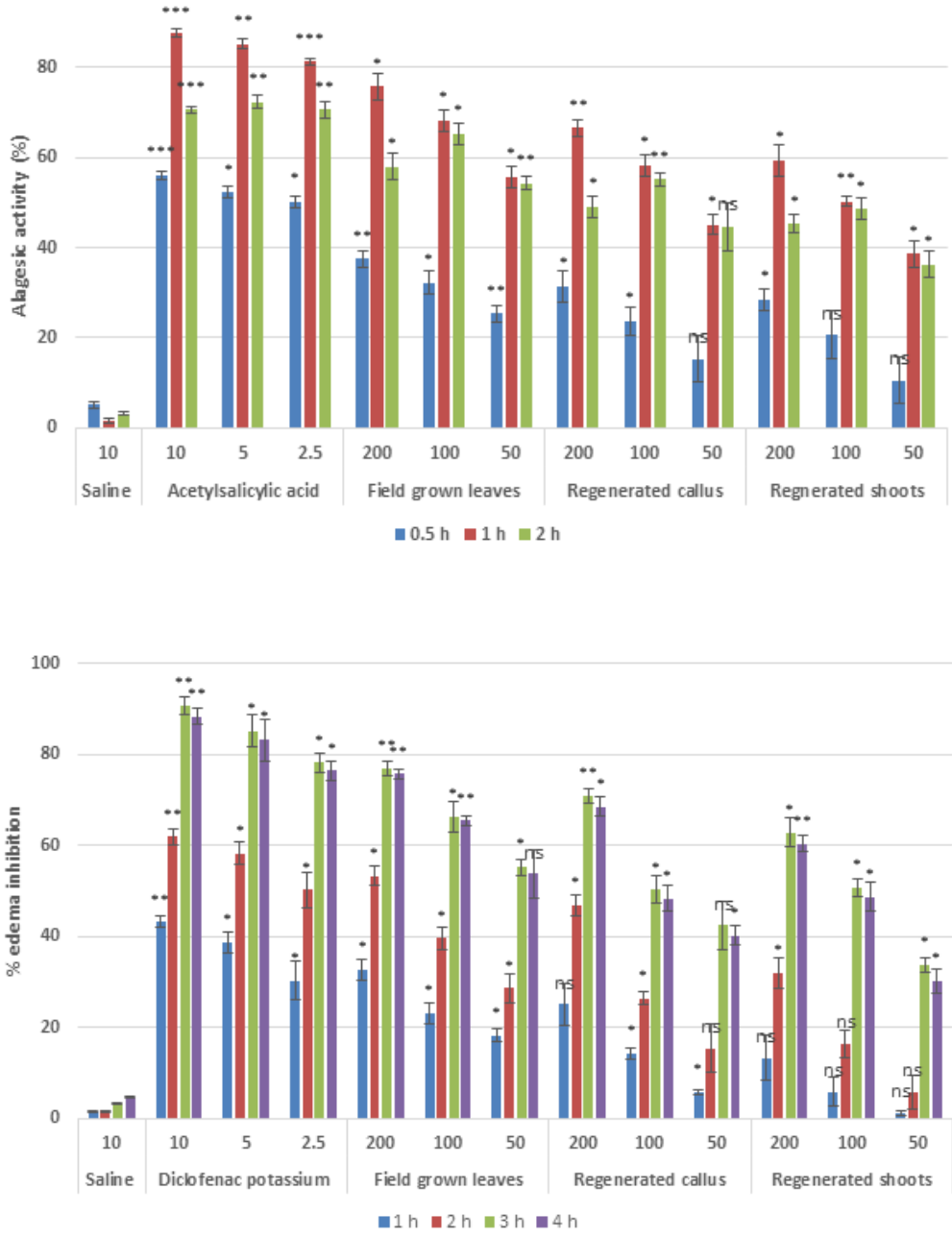

Figure 2. Analgesic activity **(A)** and edema inhibition **(B)** of lettuce Iceberg methanolic extracts.

Each value is represented as mean ± SD (n=7) with *p<0.05, **p<0.01, ***p<0.001 statistical significance as compared with saline group (10 mL/kg). Acetyl salicylic acid and diclofenac potassium (2.5 – 10 mg/kg, respectively), extracts from leaves, callus and shoots (50 – 200 mg/kg b.w.). ns: non-significance.

On the other hand, for regenerated shoots, significant and positive correlation was calculated for *in vitro* as well as *in vivo* studies except total antioxidant capacity for phenolics (p>0.05). Overall, our results showed strong significant and positive correlation with flavonoids and phenolics, which suggest their involvement in various important medicinal characteristics such as antioxidant, an-

algesic and anti-inflammatory activities. This is in accordance with the previous reports in which phenolics and flavonoids have been connected with antioxidative potential in biological systems, due to their capability of quenching free-radicals and singlet oxygen (Kim et al., 2006). Double bond conjugation, the presence of hydroxyl, methoxy or ketonic groups have been found responsible for the antioxidant action of phenols (Afshar et al., 2012). As the highest flavonoid and phenolic contents were calculated in the methanolic extract, it can be considered as the potentially best antioxidant, analgesic and anti-inflammatory extract of Iceberg lettuce.

CONCLUSIONS

The results of this study showed that field-grown plants are comparably rich source of secondary metabolites, which shows multiple pharmacological activities in a significant way. In comparison, callus of *in vitro* cultures of Iceberg lettuce showed antioxidant, analgesic and anti-inflammatory activities more than regenerated shoots. The present work signifies the importance of both field-grown and *in vitro* cultures especially callus as a valuable source of medicinally important secondary metabolites.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Table 3. Correlation between plant activities and phytochemicals of lettuce Iceberg extracts.

Plant extract	Correlation R^2	
	Total phenolic content	**Total flavonoid content**
Field-grown leaves		
Total reducing power	0.9511*	0.9221*
Total antioxidant capacity	0.9108*	0.9654*
DPPH free radical scavenging assay	0.9709*	0.9165*
Analgesic assay	0.9256*	0.9442*
Anti-inflammatory assay	0.9332*	0.9519*
Regenerated callus		
Total reducing power	0.9616*	0.8948
Total antioxidant capacity	0.8057	0.7083
DPPH free radical scavenging assay	0.9198*	0.9636*
Analgesic assay	0.9391*	0.9451*
Anti-inflammatory assay	0.9256*	0.9308*
Regenerated shoots		
Total reducing power	0.9405*	0.999***
Total antioxidant capacity	0.8194	0.9467*
DPPH free radical scavenging assay	0.9918**	0.9042*
Analgesic assay	0.9212*	0.9136*
Anti-inflammatory assay	0.9012*	0.9378*

Values are expressed as mean (n=3) and *p<0.05, **p<0.01, ***p<0.001 represent correlation significance.

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AUTHOR CONTRIBUTION:

Contribution	Younus I	Ismail H	Rizvi CB	Dilshad E	Saba K	Mirza B	Waheed MT
Concepts or ideas		x		x	x	x	x
Design	x	x				x	x
Definition of intellectual content	x		x		x	x	x
Literature search	x		x	x			
Experimental studies	x	x	x		x		
Data acquisition	x			x	x	x	x
Data analysis	x	x	x			x	x
Statistical analysis	x	x			x		
Manuscript preparation		x	x	x	x	x	x
Manuscript editing	x			x	x	x	x
Manuscript review	x	x	x	x	x	x	x

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