MICROPROPAGATION, AGRO-ENVIRONMENTAL STUDIES AND PHARMACOLOGICAL ACTIVITIES OF SAFFRON (*Crocus sativus* L.) GROWN IN CONTROLLED ENVIRONMENTAL CONDITIONS

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FACULTY OF SCIENCE
UNIVERSITI MALAYA
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THESIS SUBMITTED IN FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

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Field of Study: **BIOTECHNOLOGY**

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MICROPROPAGATION, AGRO-ENVIRONMENTAL STUDIES AND PHARMACOLOGICAL ACTIVITIES OF SAFFRON (*Crocus sativus* L.) GROWN IN CONTROLLED ENVIRONMENTAL CONDITIONS

ABSTRACT

Saffron is derived from three stigmatic lobes of *Crocus sativus* flowers, an autumn-flowering herb that is propagated solely through clonal corm multiplication. Despite its growing market demand and potential value, the saffron yield is low due to its sterile nature and distinctive eco-physiological characteristics. This study aims to explore *in vitro* cormlet formation, the effect of agro-environmental factors and gamma irradiation on saffron growth indices in Malaysia and its potential nutraceutical importance of different parts of the saffron plant. It was observed that treatment of corms with 70% ethanol followed by washing in 40% clorox and 0.2% $HgCl_2$ ensured clean aseptic viable cultures. MS enriched with BAP (4 mg/l) and IAA (1 mg/l) resulted in maximum buds sprouting response and multiple shoot primordia. Further sub culturing of shoot primordia in BAP (6 mg/l) and IAA (0.5 mg/l) was superior for achieving multiple shoots proliferation. MS supplemented with GA_3 (4 mg/l) and 4% sucrose guaranteed highest cormlet number and cormlet weight, whereas GA_3 (2 mg/l) and 2% sucrose gave highest root number and root length. In an attempt to assess the response of saffron growth and yield components to temperature, mother corm size, type of fertilizer and planting depth during the first two years, it was shown that lower temperature of 18 °C, bigger sized of planted corms and cattle manure application were the most favorable conditions for the entire reproductive and vegetative attributes of saffron irrespective of planting depth. Likewise, different doses of γ-rays (0, 10, 20, 30, and 40 Gy) was evaluated for their potential effect on morpho-agronomic characteristics of saffron and the results indicated that 10 Gy treatment stimulatively effected on various vegetative attributes, whereas

radiation at 20 Gy was the most optimal dose for the reproductive traits of saffron. Also, one of the 20 Gy plants showed four irregular dark petals, tetrafid stigma, and two stamens compared with six normal-sized petals, trifid stigma, and three stamens in control. Conversely, higher doses of 30 Gy and 40 Gy were lethal by showing deleterious effects in terms of saffron growth indices. Saffron parts i.e corm, leaf, petal and stigma obtained were further evaluated for their toxicity and pharmacological activities in mice. During the 14 days oral toxicity study, none of the saffron ethanolic extracts at a dose of 2000 mg/kg body weight showed any mortality and abnormality in all selected parameters in mice of both sexes as compared with their respective control groups. Stigma and petal ethanolic extracts showed significant enhancement behavior in carrageenan induced hind paw edema assay and hot plate analgesic assay as well as antidepressant test and anticoagulant test in mice. However, leaf and corm ethanolic extracts displayed mild activity. Likewise, SEE followed by PEE exhibited antihyperglycemic effect and showed strong hypolipidemic response in STZ-induced diabetic mice. The SEE and PEE treatment to diabetic mice also significantly restored the level of serum glucose, kidney and liver biomarkers towards the normal control which validate its use as an antidiabetic drug in folk medicine.

Keywords: Acute toxicity, antidiabetic, *Crocus sativus*, gamma irradiation, micropropagation

MIKROPROPAGASI, KAJIAN AGRO- PERSEKITARAN DAN AKTIVITI FARMAKOLOGI SAFFRON (*Crocus sativus* L.) YANG DITANAM DALAM KEADAAN PERSEKITARAN TERKAWAL

ABSTRAK

Saffron berasal daripada tiga lobus stigmatik bunga *Crocus sativus*, herba berbunga musim luruh yang dibiakkan hanyamelalui pendaraban corm klonal. Walaupun permintaan pasaran yang semakin meningkat dan nilai potensinya, penghasil saffronn adalah rendah kerana sifat steril semulajadi dan ciri karakteristik eko-fisiologi yang tersendiri. Kajian ini bertujuan untuk meneroka pembentukan kormlet in vitro, kesan faktor agro-persekitaran dan penyinaran gamma ke atas indeks pertumbuhan saffron dan potensi kepentingan nutraseutikal bahagian berlainan tumbuhan ini. Diperhatikan bahawa rawatan corms dengan 70% etanol diikuti dengan mencuci dalam 40% klorox dan 0.2% $HgCl_2$ memastikan kultur berdaya aseptik yang bersih. MS diperkaya dengan BAP (4 mg/l) dan IAA (1 mg/l) menghasilkan tindak balas percambahan tunas maksimum dan primordia pucuk berganda. Subkultur selanjutnya bagi primordia pucuk dalam BAP (6 mg/l) dan IAA (0.5 mg/l) adalah lebih baik untuk mencapai percambahan berbilang pucuk. MS ditambah dengan GA_3 (4 mg/l) dan 4% sukrosa menjamin bilangan cormlet dan berat cormlet tertinggi manakala, GA_3 (2 mg/l) dan 2% sukrosa memberikan nombor akar dan panjang akar tertinggi. Untuk menilai tindak balas pertumbuhan saffron dan komponen hasil kepada suhu, saiz pokok induk, jenis baja dan kedalaman penanaman dalam tempoh dua tahun pertama, ditunjukkan bahawa suhu yang lebih rendah iaitu 18 °C, saiz umbi yang ditanam dan baja lembu yang lebih besar. permohonan adalah keadaan yang paling sesuai untuk keseluruhan sifat pembiakan dan vegetatif saffron tanpa mengira kedalaman penanaman. Begitu juga, dos sinar-γ yang berbeza (0, 10, 20, 30, dan 40 Gy) telah dinilai untuk kesan potensinya terhadap ciri-ciri morfo-agronomi saffron dan

keputusan menunjukkan bahawa sinaran 10 Gy secara stimulatif memberi kesan pada pelbagai sifat vegetatif manakala, sinaran pada 20 Gy adalah dos yang paling optimum untuk sifat pembiakan saffron. Juga, salah satu daripada 20 tumbuhan Gy menunjukkan empat kelopak gelap tidak teratur, stigma tetrafid, dan dua benang sari berbanding enam kelopak bersaiz normal, stigma trifid dan tiga benang sari dalam kawalan. Sebaliknya, dos 30 Gy dan 40 Gy yang lebih tinggi adalah maut dengan menunjukkan kesan buruk dari segi indeks pertumbuhan saffron. Bahagian saffron iaitu corm, daun, kelopak dan stigma yang diperolehi seterusnya dinilai untuk ketoksikan dan aktiviti farmakologi dalam tikus. Semasa kajian ketoksikan oral selama 14 hari, tiada satu pun daripada ekstrak etanol saffron pada dos 2000 mg/kg berat badan menunjukkan sebarang kematian dan keabnormalan dalam semua parameter terpilih pada tikus kedua-dua jantina berbanding dengan kumpulan kawalan masing-masing. Stigma dan ekstrak etanol kelopak menunjukkan tingkah laku peningkatan yang ketara dalam ujian edema kaki belakang akibat karagenan dan ujian analgesik plat panas serta ujian antidepresan dan ujian antikoagulan pada tikus. Walau bagaimanapun, ekstrak etanol daun dan corm menunjukkan aktiviti ringan. Begitu juga, SEE diikuti oleh PEE menunjukkan kesan antihiperglisemik dan menunjukkan tindak balas hipolipidemik yang kuat dalam tikus diabetes yang disebabkan oleh STZ. Rawatan SEE dan PEE kepada tikus diabetes juga telah memulihkan tahap glukosa serum, buah pinggang dan biomarker hati ke arah kawalan normal yang mengesahkan penggunaannya sebagai ubat antidiabetik dalam perubatan tradisional.

Kata kunci: Ketoksikan akut, antidiabetik, *Crocus sativus*, penyinaran gamma, pembiakan mikro

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Asif Khan

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Ha	:	Hectare
%	:	Percentage
°C	:	Degree Celsius
2,4-D	:	2,4-dichlorophenoxyacetic acid
^{60}Co	:	Cobalt 60
AA	:	Ascorbic acid
AC	:	Activated charcoal
ANOVA	:	Analysis of variance
B5	:	Gamborg medium
BAP	:	6-Benzylaminopurine
Bas	:	Basophils
$C_{10}H_{14}O$	:	Safranal
$C_{44}\ H_{64}\ O_{24}$	:	Crocin
$C_6H_{26}O_7$	:	Picrocrocin
$CaCO_3$	:	Calcium carbonate
CEE	:	Corm ethanolic extract
CH	:	Casein hydrolysate
CM	:	Coconut milk
CO_2	:	Carbon dioxide
CRC	:	Colorectal cancer
CVD	:	Cardiovascular disorder
DCN	:	Daughter corm number
DCW	:	Daughter corm weight
DMRT	:	Duncan multiple range test

DMSO	:	Dimethyl sulfoxide
EDTA	:	Ethylene diamine tetra acetic acid
Eos	:	Eosinophils
FDW	:	Flower dry weight
FFW	:	Flower fresh weight
FST	:	Forced swim test
G	:	Gram
H	:	Hour
H&E	:	Hematoxylin-eosin
Hb	:	Hemoglobin
HCC	:	Hepatocellular carcinoma
HCl	:	Hydrochloric acid
HCT	:	Hematocrit
HDL	:	High-density lipoprotein
$HgCl_2$	:	Mercuric chloride
i.p	:	Intraperitoneal
IAA	:	Indole-3-acetic acid
IBA	:	Indole-3-butyric acid
Kg	:	Kilogram
Kn	:	Kinetin
LDL	:	Low-density lipoprotein
LEE	:	Leaf ethanolic extract
LS	:	Linsmaier and Skoog
Lym	:	Lymphocytes
MCH	:	Mean corpuscular hemoglobin
MCHC	:	Mean corpuscular hemoglobin concentration

MCV	:	Mean corpuscular volume
Min	:	Minute
Mon	:	Monocytes
MS	:	Murashige and Skoog
N6	:	Nitsch medium
NaOCl	:	Sodium hypochlorite
Neu	:	Neutrophils
PEE	:	Petal ethanolic extract
PGR	:	Plant growth regulator
Plt	:	Platelet
RBC	:	total red blood cell
RDW	:	Red blood cell distribution width
S	:	Second
SDW	:	Stigma dry weight
SEE	:	Stigma ethanoic extract
SFW	:	Stigma fresh weight
STZ	:	Streptozotocin
TC	:	Total cholesterol
TG	:	Triacylglycerol
v/v	:	Volume per volume
w/v	:	Weight per volume
WBC	:	White blood cell
Zn	:	Zeatin

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INTRODUCTION

1.1 INTRODUCTION

Saffron (*Crocus sativus*) is a herbaceous plant grown as a source of its spice for nearly 3,500 years. It belongs to the large Iris family Iridaceae (Liliales, Monocots) whose genomes size is quite large and poorly characterized. Among all the 88 species of the genus *Crocus*, saffron (*Crocus sativus* L.) is given prime importance due to its unique characteristics which distinguish it from other agricultural plants and is also considered to be the world's priciest spice. According to a certain estimate, it takes between 70,000 -200,000 flowers to produce 1 kg of saffron which equates to 370-470 h of work (Tsaftaris et al., 2013). Currently, saffron is mainly cultivated in Iran, India, Spain, France, Greece, Afghanistan, Azerbaijan, Turkey, and Pakistan with a global annual production of approximately 301 ton per year.

The genetic origin of saffron is not yet completely clear. However, based on biomolecular analysis, two different hypothesis exist as to its origin: by autopolyploid from a wild diploid *Crocus*, such as *C. cartwrightianus* (Grilli-Caiola et al., 2004), or through allopolyploid by the fertilization between *C. hadriaticus* and *C. cartwrightianus* (Castillo et al., 2005). The area of origin of saffron is obscure, but it is said to have been originated from Crete and from there became prevalent in India, China and throughout Mediterranean Europe (Negbi, 1999).

In general, due to cytological impairments, such as triploidy and self-incompatibility mechanisms, saffron is assumed to be an almost completely sterile triploid crop. Hence, saffron propagates solely through annual renewal daughter corms, produced by the mother corms. In other words, each progeny corm acts as a mother corm for the subsequent growing seasons. Additionally, each mother corm develops into only 4-5 replacement cormlets in one growing season, hence the rate of natural propagation of

corms is very low. Moreover, different pathogens such as viruses, fungi and bacteria attack the corm and cause rotting and necrosis thereby decrease the yield and quality of saffron (Plessner et al., 1990). Therefore, an alternative method that can produce pathogen-free corms rapidly and efficiently must be explored for mass propagation of saffron.

Besides conventional methods of propagation, many bulbous and cormous monocotyledonous plants are successfully grown using *in vitro* culture techniques within the last few decades. Many economically important monocot species with ornamental, medicinal, and nutritional importance have been used for *in vitro* clonal multiplication and secondary metabolites production (Ascough et al., 2009; Rogers, 2003). Therefore, micropropagation has been suggested as the best alternative technique for mass multiplication of saffron. The successful tissue culture of saffron for the first time was reported by Ding et al. (1979). Organogenesis either Direct (Bhagyalakshmi, 1999; Lapadatescu et al., 2013) or Indirect (Chen et al., 2003; Lapadatescu et al., 2013) and somatic embryogenesis (Blazquez et al., 2004; George et al., 1992) from differentiated tissues of *Crocus sativus* is also reported in the literature.

Flower emergence and productivity in saffron is controlled by several factors, such as fertilizers, temperature, water availability, corm size, soil nutrients and planting depth. However, previous studied have shown that temperature is the main factor affecting flowering in saffron (Koocheki et al., 2010; Molina et al., 2004; Molina et al., 2005). Generally, nutrient reserves in mother corm regulate saffron growth, especially during early stages of growth. In other words, bigger corm supplies more energy to the replacement corms and subsequently, improve plant growth and productivity (Koocheki & Seyyedi, 2016). Besides mother corm size, soil amendments such as fertilizers and manures also favor soil conditions by enhancing water-storage capacity of soil, the pH in

acid soils, infiltration rate and hydraulic conductivity, and reducing soil bulk density which in turn significantly improve the qualitative and quantitative traits of saffron (Koocheki & Seyyedi, 2015; Yarami & Sepaskhah, 2015). Manure is a good nutrient source for plants containing most or all of the 14 soil-derived plant mineral nutrients that improves soil structure (Yarami & Sepaskhah, 2015). The optimal planting depth is also among one of the most critical effective strategies for enhancing yield of saffron during the first and subsequent years (Bayat et al., 2016). However, the growth and flowering response of saffron to planting depth is least studied.

Genetic variation serves as the raw material for plant breeding programs and new variation is necessary to add desirable traits in breeding practices. However, in instances wherein a particular genetic trait is immediately unavailable to be crossed into breeding materials in a crop species, the genetic variation can be developed through an alternative mean such as induced mutation. Induced mutation has been achieved for decades in plants. Mutation can be induced by many ways but the three major means of mutations are radiations, chemicals and transposon insertion (Holme et al., 2019). The advantage of induced mutation over natural mutation is that the genetic variation for genetic enhancement and breeding is created in a short period of time (Horn et al., 2016). The first induced mutations in plants were generated in 1928 by exposing corn and barley plants to X-rays (Ulukapi & Nasircilar, 2018). Other types of irradiations that have shown promising results in crops are fast neutron bombardment and gamma irradiation. These treatments can induce changes in a single nucleotide (point mutations) or loss of a chromosomal segment (deletions). It is reported that many plant traits such as disease resistance, seed shattering resistance, plant height, seed maturity, oil quality and quantity, malting quality as well as quality and size of starch granules have been successfully modified through induced mutagenesis (Horn et al., 2016).

The most important feature in mutation research is to evaluate the appropriate dose for the species. For such studies, the first generational seedlings (M1) are compared to untreated seedlings in terms of plant growth attributes and survival percentages (Ulukapi & Ozmen, 2018). Keeping in view the importance of the valuable spice and no up to date research on the gamma irradiation sensitivity of saffron, it is absolutely necessary to carry out a dose determination study before largescale studies are carried out.

Conventionally, saffron has a long history of being used as a spice and food additive due to its flavor (picrocrocin), colorant (crocin), and aroma (safranal) features that are confined in the three-branch stigma of the *C. sativus* flower (Melnyk et al., 2010). Saffron tea has been used as a potential complementary treatment for psoriasis in medical nutrition therapy (Hosseinzadeh & Nassiri-Asl, 2013). Besides its use in cosmetic preparation and coloring industries, it has also been used as a drug to cure many human disorders such as coughs, colds, insomnia, colic, cramps, asthma and bronchospasms, liver diseases and epilepsy (Gohari et al., 2013; Hosseinzadeh & Nassiri-Asl, 2013). Various studies showed that saffron and its constituents possess antimicrobial (Vahidi et al., 2010), antitumor (Bolhassani et al., 2014), anti-inflammatory, antioxidant, immunomodulatory (Boskabady & Farkhondeh, 2016), antidiabetic (Samarghandian et al., 2014), antidepressant (Hosseinzadeh & Nassiri-Asl, 2013), anti-hypertensive (Llorens et al., 2015) and antiplatelet (Arimitsu et al., 2014) effects.

All the above desirable properties of saffron have been attributed to the stigmas, whereas other parts of the plant have been much less studied. Bioactive compounds extracted from petal have shown antinociceptive, anti-inflammatory (Hosseinzadeh & Younesi, 2002), antibacterial (Asgarpanah et al., 2013), anticancer (Hosseinzadeh et al., 2005), antidiabetic (Hemmati et al., 2015), anti-obesity (Hoshyar et al., 2016), antidepressant (Basti et al., 2007), anti-tyrosinase and free radical scavenging properties

(Li et al., 2004) as well as reduce blood pressure and contractility (Fatehi et al., 2003). Several phenolic compounds from saffron leaves have been identified showing antibacterial (Jadouali et al., 2018), anticancer (Sánchez-Vioque et al., 2016), antioxidant and metal ion chelating properties (Sánchez-Vioque et al., 2012). Bioactive constituents in saffron corm such as proteoglycans revealed to have a cytolytic effect on human tumor (Escribano et al., 2000) and plant (Fernández et al., 2000) cells, and triterpenoid saponins on fungicidal (Rubio-Moraga et al., 2013) and anticancer (Rubio-Moraga et al., 2011) activities. Polyphenols showing radical scavenging activity (Baba et al., 2015) and a mannan-binding lectin (Escribano et al., 2000) have also been investigated in corm.

Nevertheless, a large quantity of saffron by-products is produced during the stigmatization process with little commercial value but thrown away after harvesting. According to an estimate, approximately 1500 kg of leaves, 350 kg of petals, and several hundred cormlets too small for cultivation or biologically and physically damaged to be regrown are rejected to get spice of only 1 kg. However, this biomass contains a multitude of phytochemical ingredients whose exploitation would significantly enhance the sustainability and profitability of saffron yield. Beside stigma, these saffron based by-products also need to be tested to assess its pharmacological applications.

1.2 Rationale of present study

The application of tissue culture practices offers great potential to overcome the challenges in mass propagation of saffron. In the earlier studies conducted on micro-propagation of saffron, main emphasis of the experimental research was merely placed on plantlet production (Bhagyalakshmi, 1999; Igarashi & Yuasa, 1994; Majourhat et al., 2006). The hardening of *in vitro* grown plantlet is generally difficult with a very low survival rate. Therefore, the production of *in vitro* raised cormlets is an added advantage as cormlets are easy to handle and convenient for field transfers. There are many studies

reported on cormlets production either directly from apical or axillary buds (Aguero & Tizio, 1994; Karaoglu et al., 2006; Milyaeva et al., 1995; Mir et al., 2010; Plessner et al., 1990) or indirectly with an intermediate callus phase (Ahuja et al., 1993; Dhar & Sapru, 1993; Piqueras et al., 1999) However, all these findings are fragmentary and the protocols are not efficient to be used as routine procedure or for commercialization.

Even with its prominence in the market, saffron production is running low because of fewer growing countries, limited areas of cultivation, smaller amount of yield and conventional management and processing techniques (Gresta et al., 2008b). Interestingly, the researchers have recently started focusing on saffron production at larger scale to cope with its increased demand and small yield. Modern studies reveal that a number of agronomic and environmental factors play a fundamental role in achieving optimal yield and production of saffron which affect its quantitative and qualitative parameters such as temperature, soil, photoperiod, planting time, and fertilizers (Ahrazem et al., 2015; De Juan et al., 2009; Kumar et al., 2009). Besides, mutation breeding has proven to be a highly effective method for improving crops and creating new genetic resources. Gamma radiation is one of the vital physical agents used for the improvement of the productivity of many crops. Furthermore, irradiation has proven an adept means of encouraging the expression of recessive genes and producing new genetic variations. Since, flower is the most valuable part of saffron, understanding the interaction of saffron with these agro-environmental factors along with gamma irradiation is necessary to optimize flower yield and quality of saffron.

Many bioactive and health-promoting properties have been investigated both *in vitro* and *in vivo* in saffron but it is limited to saffron stigma and other plant parts such as petal, leaves and corms have not received much attention. To the best of our knowledge, there is no report on the pharmacological activities of *in vivo* grown saffron plants. Therefore,

an *in vivo* investigation on the biological activities of *in vivo* grown saffron plants is needed.

The aim of this study was to describe *in vitro* cormlet formation, the effect of agro-environmental factors and gamma irradiation on flower formation and the pharmacological importance of the different parts of saffron plant grown under controlled environmental conditions.

1.3 Objectives of the Study

The specific objectives of the study were:

1. To study the effect of different media (MS, B5) and various combinations of growth hormones (NAA, IAA, Kn, BAP) on *in vitro* regeneration and cormlet formation of various explants of saffron.
2. To determine the effect of agronomic (type of fertilizer, corm size and planting depth) and environmental (temperature) factors on growth and flowering of saffron in two harvesting seasons.
3. To study the effect of gamma radiations on growth and flowering of saffron.
4. To evaluate the acute toxicological study of *in vivo* grown saffron ethanolic extracts in mice.
5. To assess the analgesic, anti-inflammatory, anticoagulant, antidepressant, and antidiabetic effects of saffron ethanolic extracts in mice.

LITERATURE REVIEW

2.1 General Introduction to the Genus *Crocus*

The genus *Crocus*, belonging to the family Iridaceae, embraces approximately 85 species of flowering plants. Genus *Crocus* is distributed between 10°W and 90°E and between 30°N and 52°N (Mykhailenko et al., 2019). Most *Crocus* species are phytogeographically associated with the Mediterranean region's floristic zone, with some also having broad distributions into the Irano-Turanian phytochorion (Erol et al., 2014). *Crocus spp.* is found in environments with an ideal temperature of 4 to 23 °C, a median yearly precipitation of 0.1–1.1 m, and soil that drains well (pH 5.8-7.8). *Crocus* species are distinguished by their thin grassy leaves with yellow, white or purple flowers; plants are primarily native to the Mediterranean basin (Mykhailenko et al., 2019). Amongst the 85 species of genera *Crocus*, *C. sativus* produces the world's costliest agricultural product, saffron (Gohari et al., 2013).

The life cycle of a *Crocus* starts with seed germination followed by various stages of seedling in the first year that leads to the mature plant after 3 to 5 years. The autumn-flowering crocuses typically germinate between September and November, whereas the flowering time of vernal crocuses depend on climatic conditions and habitat but usually flower between mid-winter to spring. Crocus seeds have a lengthy germination capacity and many only germinate after an year or even more years dormant in their native environment (Kerndorff et al., 2015).

In *Crocus* the mechanism of pollination and fertilization is very complex. In any case, it completed the life cycle of the *Crocus* by providing the prerequisite for the growth and development of seeds. Typically, insects such as beetles, flies and bees accomplish the pollination, making this genus entomophilic or zoophilic. The most important features attributes to zoophilic plants are that they are angiosperms bearing hermaphrodite

flowers, strong fragrance, prominent colors, nectar or pollen for nourishment and well-marked surfaces of pollen with high quantity of pollen cement. However, minimal, or average quantity of pollen cement is present in genus *Crocus* with absence of strongly marked surfaces of the pollen. They are either inaperturate or weakly to sometimes strongly spiraperturate (Kerndorff et al., 2015).

2.1.1 Taxonomy of *Crocus sativus* L.

Crocus sativus L. belongs to the Division-Magnoliophyta, Class-Liliopsida, Order-Asparagales, Family-Iridaceae, Sub-family-Crocoideae and genus-*Crocus* as shown in figure 2.1. *Crocus* and *Crociris* are the two subgenera of the genus *Crocus*. Adding to that, *Crocus* and *Nudiscapus* are the two sections of the subgenus *Crocus*. Section *Crocus* is further divided into 6 series and *Nudiscapus* into 9 series. There are 10 species in the Series *Crocus* but only *C. sativus* produces the spice, saffron and has similar botanical descriptions globally. However, a few synonyms such as *C. sativus* var. *orsinii, C. officinalis* var. *sativus* Huds., *C. autumnalis* Smith*, C. sativus* var. *cashmirianus* Royle, *C. sativus* var. *officinalis* Linn., and *C. orsinii* Parl are reported in the literature (Grilli-Caiola & Canini, 2010). Most of the *Crocus* species and sub-species are only limited to the Balkan Peninsula and Turkey. Turkey's flora comprise a total of 32 species, of which 18 are endemic (Coşkun et al., 2010) while according to another study, *Crocus* is characterized by 36 species in Turkey (35 of them being endemic) (Kültür & Aslan, 2009). Greece alone contributed around 40% of the world's wild *Crocus* diversity (Ahrazem et al., 2015). Italy (10 species), Spain (6 species) and Hungary (6 species) are the other countries with *Crocus* species.

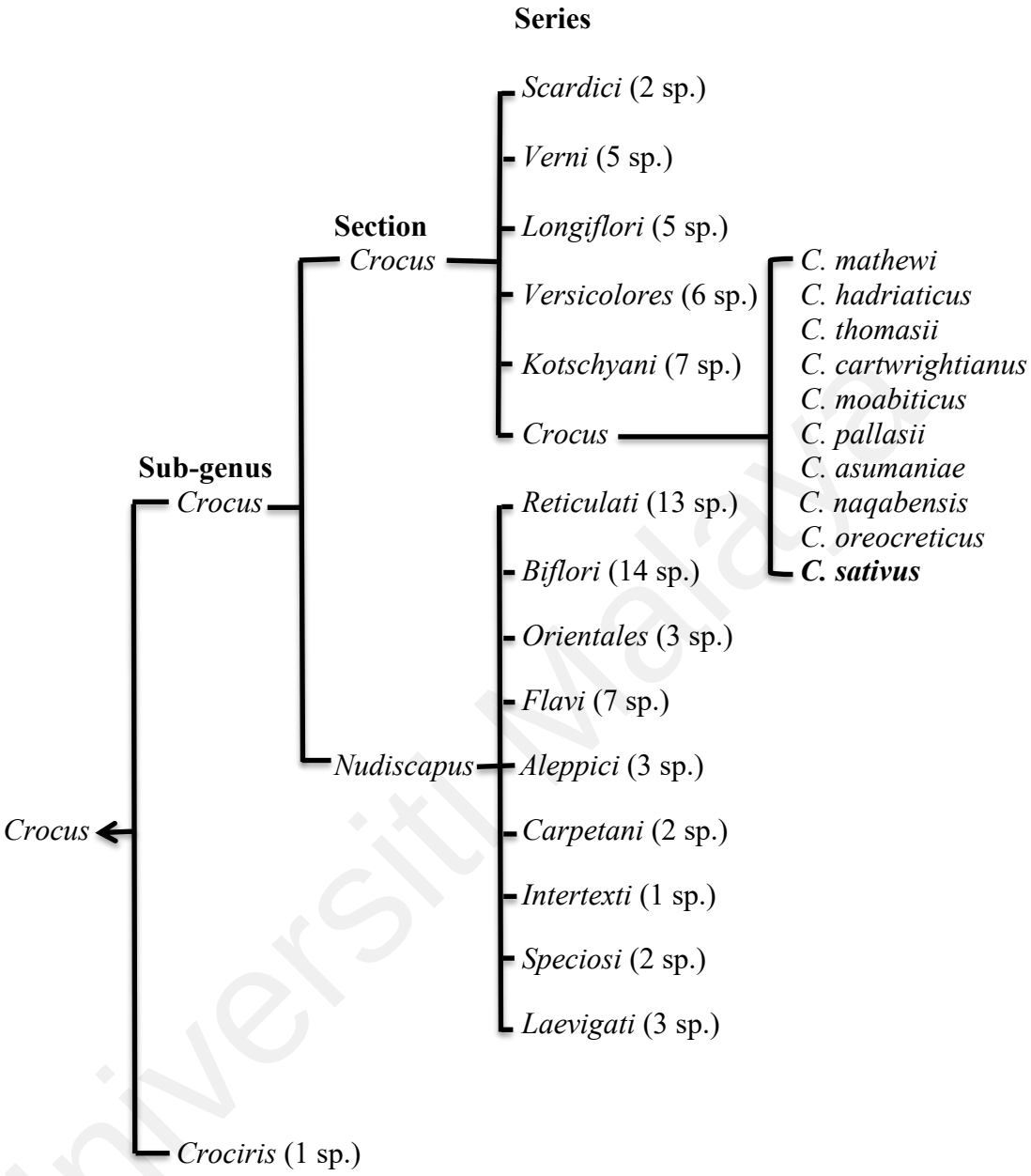


Figure 2.1: Phylogenetic tree of Genus *Crocus* (Mathew, 1982)

2.1.2 Distribution

The distribution pattern of saffron lies within the range 0°E-90°E longitudinal from Spain to Kashmir and 30°N-45°N latitude from Persia to England. Currently, saffron is cultivated in countries like Iran, India, France, Turkey, Spain, Italy, Pakistan, Greece, Morocco, Israel, Lebanon, Azerbaijan, Afghanistan, China, New Zealand, Australia, Argentina, USA, Chile and Mexico (Jalali-Heravi et al., 2010; Lone et al., 2016). Current estimates for total world annual production of saffron are 301 ton per year. Iran, the world's largest producer of saffron with around 87,924 ha harvest area, is said to produce more than 90% of the total saffron i.e. 280.6 ton (Ghorbani & Koocheki, 2017). Khorasan province alone occupies 46000 ha accounting for 137 ton per year of the total production (De Juan et al., 2009). Indian occupied Kashmir with cultivation area and saffron production of 3785 ha and 11 ton respectively, is the second largest contributor of saffron (Husaini, 2014). Global production of saffron is given in Table 2.1.

Over the last few decades despite a gradual increase in its price, saffron production has been reduced in many traditional cultivated countries, such as England, Italy, Spain, and Greece. Spain for example, was once the leading cultivator of saffron and nowadays it makes only 0.3 to 0.5 ton saffron. One of the main reasons for the decline of this crop in these traditionally producing regions is its small harvesting area. In Spain, the total cultivated area for saffron in 1971 was 6,000 ha that have been dropped to 116 ha in 2006 (De Juan et al., 2009), whereas in Greece from 1600 ha in 1982 to 860 ha and from 300 ha in 1910 to 6 ha in central Italy (Fernandez, 2004). Likewise, in the past decade a considerable reduction of 83%, 215% and 72% in area, production and yield of saffron is reported in Kashmir, with an annual drop of 7.5% in area than annual growth of 11.5% in Iran during the same period (Kirmani et al., 2014).

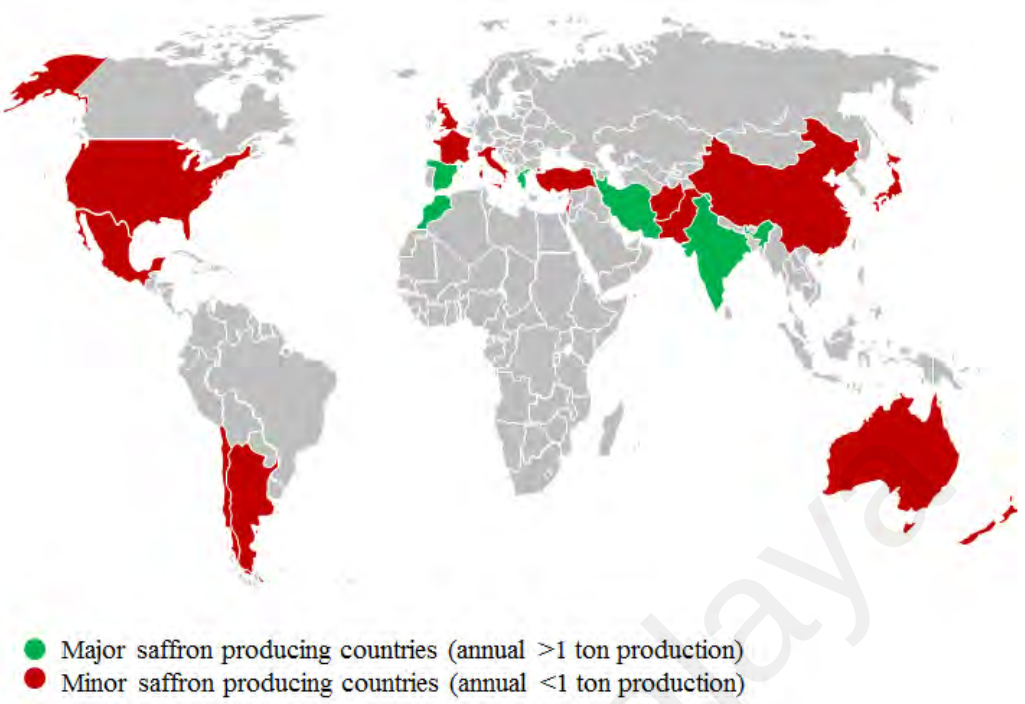


Figure 2.2: Minor and major saffron producing countries

Table 2.1: Global saffron production

Country	Area (ha)	Production (t)	Yield (kg ha^{-1})	Reference
Iran	87,924	280.6	3.20	Ghorbani & Koocheki (2017)
India	3785	11	3.44	Husaini (2014)
Morocco	1600	3.5	2.19	Mzabri et al. (2017)
Greece	860	4-6	4.65-6.97	Melfou et al. (2015)
Azerbaijan	675	-	-	Azizbekova & Milyaeva (1999)
Afghanistan	250	0.6-0.8	2.4-3.2	Mollafilabi & Aslami (2010)
Spain	116	1.33	11.4	De Juan et al. (2009)
Italy	35	0.12	3.43	Gresta et al. (2008b)
Turkey	-	0.01	-	Thiercelin (2004)

2.1.3 Morphology and Biology

Saffron is an autumn flowering perennial herb with sub hysteranthous behavior, as it blooms before (hysteranthy), at the same time (synanthy), or after leaf appearance (Gresta, F. et al., 2008b). Saffron plant comprises of corm, foliar structure, and floral organs as the main parts as shown in figure 2.3. The saffron corm is an underground stem which consists of nodes and is covered by tunics. These corms vary in size from 3-5 cm in diameter and are similar to gladiolus corms. Each corm bears 1 (sometimes 2) apical bud and about 4-7 lateral buds, arranged irregularly in a spiral form. Corms produced from apical buds are bigger in size than corms formed by lateral buds (Gresta et al., 2008b). Saffron corms have two different types of structural and functional roots system viz., fibrous (absorbing) roots and contractile roots. The fibrous roots arise at the base of the mother corm. These roots are thin and straight with an ability to absorb water and nutrients. On the other hand, the contractile roots, thicker than the fibrous roots, arise at the base of the lateral buds. These roots have the capability to move into the ground so that the corm maintain depth in the soil (Choi et al., 1996). Each saffron corm forms 6-15 erect, green, grass-like leaves or monophylls that can reach up to 50 cm in length (Dhar & Mir, 1997; Fernandez, 2004). Likewise, each corm produces one to numerous or as many as 12 flowers with a large perianth of 6 violet to purple tepals connate at the base in a long slender tube (Serrano-Díaz et al., 2013). Three stamens, attach to the base of the outer segments, have short filaments and yellow basifixed anthers. The pistil comprises of an underground ovary, from which a narrow thread-like style arises which ends in triple dark red stigmas of 30-40 mm length. The stigma after drying process form the commercial spice, saffron (Gresta et al., 2008b).

Figure 2.3: Main components of saffron (A) corms (B) flower (C) stigmas

2.2 Micropropagation

According to (Murashige, 1974), micropropagation is the potential of a plant part to redevelop into a complete plant, organ, or tissue under sterile and controlled environment. Clonal propagation, *in vitro* propagation, rapid propagation, and plant tissue culture are further names for micropropagation. It is a main tool for both fundamental and applied research with many practical applications in agriculture, industries, and a prerequisite for genetic transformation in plants (Thorpe, 1990). Gottlieb Haberland, a German botanist and the father of micropropagation, for the first time isolated and cultured the plant cells on Knop's salt solution in 1898 (Krikorian & Berquam, 1969), whereas RJ Gautheret obtained true plant tissue cultures from cambial tissue of *A. pseudoplatanus* for the first time in 1983 (Gautheret, 1983).

In conventional farming methods, several plants grown under specific climatic conditions form seeds and some of which unable to germinate seeds or produce flowers. Besides, they also need longer time to grow and develop into a complete plant. On the contrary, there are many advantages of micropropagation techniques over conventional approach (Prakash & Van Staden, 2007). These techniques are cheaper and take less time and space than traditional techniques. It is also useful in the production of plants from seeds that would not otherwise germinate e.g. *Nepenthes* and orchids (Fasolo & Predieri, 1988).

Clonal propagation is based on the concept of totipotency i.e. the competence of a plant's cell or tissue to transform into a complete plant (Azman et al., 2014). The idea of totipotency compelled scientists to undergo different studies on plants which led to the development of effective plant tissue culture techniques for various crops. Different cells have different level of totipotency as the degree of differentiation and specialization of one cell vary from that of another (Rukundo, 2009).

Plants are regenerated by three different approaches of micropropagation: a) apical meristem (nodes or shoot tips) b) organogenesis (using callus), and c) somatic embryogenesis. The pathway through which a cell or tissue regenerates into a complete plant is divided into three stages: (1) shoot initiation and multiplication, (2) shoot elongation and (3) *in vitro* root formation from the shoots to form stably growing plantlets.

These tissues have a high rate of cell division and the capacity to create significant amounts of growth regulators like auxin and cytokinin. Sometimes, small-size explants exhibit less growth, whereas utilizing large-size explants increases the risk of infection (Staba, 1980).

The source of the explant i.e. position, type, size and age of that explant determines the success of plant tissue culture (Nhut et al., 2007). Different cells have different capability to regenerate into a new plant (Sasikumar et al., 2009). Although callus, flowers, roots, leaves, stems, and shoots can be used as explants but root tips, nodal buds and shoot tips are most commonly used as explants in micropropagation. These tissues have a high rate of cell division and the capability to produce significant amounts of plant growth regulators i.e., auxin and cytokinin. Moreover, small-sized explants sometimes show less growth, whereas on the other hand, large explants increase the risk of contamination (Staba, 1980).

Micropropagation is commonly used for large scale production of plants. In addition to being a research tool, its significance in the field of plant propagation, plant improvement, disease eradication and production of secondary metabolites is rapidly increasing. A single explant can be reproduced into millions of plants using the plant tissue culture approach in a relatively very short period of time and space under controlled environment all year long, regardless of the weather and season (Idowu et al., 2009).

2.2.1 Micropropagation of *Crocus sativus*

The techniques of micropropagation are practically applied on all type of plants ranging from cash crops and food to medicinal and ornamental plants and even trees. It has increased the mass propagation of a large a number of vegetatively propagated crops (Johkan et al., 2016; Lata et al., 2016; Wetzstein et al., 2018). In saffron, *in vitro* plantlets and cormlets have been formed either directly from the apical or axillary buds (Aguero & Tizio, 1994; Homes et al., 1987; Karaoglu et al., 2006; Milyaeva et al., 1995; Plessner et al., 1990) or through intermediate callus phase (Ahuja et al., 1993; Chauhan et al., 1999; Dhar & Sapru, 1993; Ding et al., 1981; Huang, 1987; Ilahi et al., 1987; Piqueras et al., 1999) or from the multiple shoots raised from the buds (Sharma et al., 2008; Zeybek et al., 2012). Information available so far regarding plantlet and cormlet formation is summarized in table 2.2.

Table 2.2: *In vitro* plantlet and cormlet formation of saffron

Explants	Medium	PGRS (mg/l)	Response	Reference
Small corm sections	MS	2,4-D (1.0), Kn (0.5)	Buds and daughter corms formation	Ding et al. (1979)
Corm	MS	IAA (1.0), NAA (1.0)	Callus formation	Ding et al. (1981)
Callus	MS	IAA (1.0), NAA (1.0)	Bud differentiation and plantlet production	
Corm	MS	2,4-D (0.5), BAP (0.5), Sucrose 5%, CM (2%)	Nodulated callusing	Ilahi et al. (1987)
Corm	MS	2,4-D (1.0), BAP (0.5), Sucrose 5%, CM (2%)	Smooth callusing	
Nodulated & smooth callus	MS	2,4-D (1.0), BAP (0.5), Sucrose 5%, CM (2%)	Shoot bud differentiation	
Nodulated & smooth callus	MS	2,4-D (0.1), BAP (0.5), Sucrose 5%, CM (2%)	Increase in shoot buds number	
Shoot buds	MS	2,4-D (0.1), BAP (0.1),	Shoots similar to natural plants	
Shoot	MS	NAA (2.0)	Rooting of shoots	
	½ MS	2,4-D (0.1), BAP (0.1)	Swelling of shoots similar to natural corms	
Corm	B5, MS	2,4-D (2.0), Sucrose 2%	Small mini-corms	Homes et al. (1987)
Mini-corm	B5, MS	2,4-D (2.0), Sucrose 2%	Shoots regeneration from cormlets produced	
Basal leaf part	N6	2,4-D (2.0), BAP (0.5)	Callus formation	Huang (1987)
Callus	MS	NAA (0.5), BAP (0.2)	Shoot bud formation	
Shoot buds	½ MS	IAA (1.0)	Shoot development	
Bulb tissue	MS	Zn (0.3), 2,4-D (0.5)	Callus formation	Isa et al. (1990)
Callus	Liquid MS	2, 4-D (1.0)	Spherical nodule	
Nodules & callus	MS	BAP (1.0), NAA (1.02)	Plantlets formation	
Corm & Bud	MS	Kn (2.0), IAA (2.0), AA (100)	Plantlet and cormlet formation	Gui et al. (1988)

Table 2.2, Continued.

Apical buds with corm tissue attached & whole corm	MS	GA3 (0.5), 2,4-D (0.5), Kn (0.5), CH (500), AC (1000), AS (160)	No rooting, leaf development decreased; dimensions of developing corm increased at 15°C; complete rooting inhibition, leaf elongation reduction and increase in corm development at 20 & 25°C; Leaf elongation at 10-15°C	Plessner et al. (1990)
Isolated apical buds	MS	Zn (3.0), 2,4-D (1.0), CH (500), AS (160)	Bud sprouting, Leaf elongation, greening and corm development	
Small corms	MS	Kn (6.0), 2,4-D (1.0), CH (500), AS (160), EG (1000)	Single minute dormant corm without leaf and root production	
Floral apices	MS	2,4-D (2.0), Kn (2.0), Sucrose 2%, AS (100)	Callus formation	Dhar & Sapru (1993)
Callus	MS	NAA (1.0), Kn (2.0), Sucrose 2%, AS (100)	Callus proliferation, Corm and shoot like structure	
Bulblets	LS	NAA (3.72), BAP (4.5)	Golden yellowish, friable & hard, compact nodular calli	Ahuja et al. (1993)
Nodular callus	LS	NAA (3.72), BAP (4.5)	Shoot bud differentiation	
Shoot buds	MS	NAA (0.93), BAP (1.13)	Increase in shoot buds number and length	
Regenerated shoots	½ MS	NAA (0.93), BAP (1.13), AC (2%)	Small globular corms with well-developed roots	
Ovary	MS	BAP (11.26), NAA (0.93-9.31)	Nodular calli and friable calli	Igarashi & Yuasa (1994)
Nodular callus	MS	BAP (0.11), NAA (0.9)	Maximum shoot regeneration	
Shoots	MS	BAP (0.11), NAA (0.9)	Rooted plantlets	
Lateral buds	N6 and MS microelements	NAA (0.01), BAP (2.32), Zn (1.51), GA3 (0.035)	Bud growth	Aguero & Tizio (1994)
	½ MS	BAP (1.49)	Shoot multiplication	
In vitro shoots	½ MS	No PGRs, Sucrose 6%	Corm & shoot formation	
Apical & lateral buds	MS	IAA (3.0), Zn (3.0), Glucose 3%	Shoot bud initiation (90%) and callus formation (80%)	Milyaeva et al. (1995)

Table 2.2, Continued.

Shoot bud	MS	IAA (3.0), Zn (3.0), Glucose 3%	Shoot formation	
Shoot	MS	IAA (3.0), Zn (3.0), Glucose 3%	Corm formation	
Regenerated microcorm	MS	IAA (3.0), Zn (3.0), Glucose 3%	Shoot & microcorm formation	
Corm cut surfaces	MS	IAA (3.0), Zn (3.0), Glucose 3%	Explants callusing & embryoid formation; shoot & corm formation from embryoids	
Corm	MS/B5	NAA (1.4), Kn (0.6) or 2,4-D (0.8)	Callus formation	Jia et al. (1996)
Callus	MS/B5	NAA (0.6-1.4)	Plantlet & corm formation	
Style	LS	BAP (1.0, 5.0), NAA (1.0)	Callusing & contractile roots, buds neoformation, corms at the base of buds	Ebrahimzadeh et al. (1996)
	LS	BAP (1.0), NAA (5.0)	Shoot regeneration, corm formation, stigma & contractile root formation	
Ovary	LS	BAP (1.0), NAA (1.0, 5.0); BAP (5.0) and NAA (10.0)	Shoot formation	
Half ovary	LS	BAP (1.0), NAA (5.0)	Shoot formation	
Petal	LS	BAP (5.0), NAA (10.0)	Buds neoformation and corms at the base of shoots	
Anther & leaf	LS	-	No response	
Corm	LS	NAA (1.0), BAP (1.0)	Plantlet regeneration, Maximum cormlet formation	
Leaf base	MS	2,4-D (1.0), Kn (1.0)	Nodular embryogenic calli	Chauhan et al. (1999)
Nodular embryogenic callus	MS	IBA (2.0), BAP (10.0)	Multiple shoot formation	
Shoot	Liquid MS	IBA (10.0), BAP (2.0)	Cormlet formation	
In vitro cormlets	MS, Liquid MS	IBA (10.0), BAP (2.0)	Rooting	
Ex vitro cormlets	MS	IBA (2.0), BAP (10.0)	Multiple shoot formation	
Meristematic corm tissue	MS	2,4-D (0.1), BAP (2.0)	Nodular cormogenic calli	Piqueras et al. (1999)

Table 2.2, Continued.

Cormogenic callus	Liquid MS	2,4-D (0.1), BAP (2.0)	Multiplication of cormogenic nodules, fresh weight increased two folds compared to MS medium	
Cormogenic callus	MS	BAP (5.0)	Induction and shoot differentiation	
Cormogenic callus	MS, Liquid MS	2,4-D (0.1), BAP (2.0), Sucrose 3, 5, 7%	Decrease in adventitious shoot induction and nodular calli proliferation, more fresh weight of liquid medium cultures	
Cormogenic callus	MS	2,4-D (0.1), BAP (2.0), NaH_2PO_4 (50.0-150)	Growth and shoot development in cormogenic calli	
Cormogenic callus	MS	2,4-D (0.1), BAP (2.0), L-glutamine (120)	Enhanced rate of adventitious shoot induction and multiplication	
Cormogenic callus	MS	2,4-D (0.1), BAP (2.0), CH (120)	Positive effect on callus induction and multiplication	
Cormogenic callus	MS	2,4-D (0.1), BAP (2.0), PBZ (0.1)	Enhanced adventitious shoot induction	
Cormogenic callus	MS	2,4-D (0.1), BAP (2.0), Imazalil (0.5)	Maximum shoot number per explant	
Nodules with adventitious shoots	MS	No PGRs	Nodules produced normal roots and leaves with basal microcorm	
Ovary with stigma	MS	NAA (5.0), BAP (1.0), L-glutamine (400), AA (100), Sucrose 4.5%	Shoots cluster and elongated structures from the base of ovary	Bhagyalakshmi (1999)
Shoot buds	MS salt and (Novak et al., 1989) vitamins	Sucrose 2%	Maximum number and length of leaves	
Shoots	MS	BAP (0.5), NAA (0.1)	Increased shoot growth by increasing leaf number and length	
Corms	MS	NAA (0.5), BAP (2.0)	Single shoot development from explants with eye	Karaoglu et al. (2006)
Shoots	MS	NAA (0.5), BAP (2.0)	Dormant cormlets regeneration from main explant after 24 weeks	

Table 2.2, Continued.

Cormlets	MS	NAA (0.5), BAP (2.0)	Dormancy breakdown 44 weeks after culturing with regeneration of 2-3 cormlets	
Developing corms	MS	NAA (0.5), BAP (2.0)	Shifted to pots for onward shifting to green house	
Mature flower base	MS	NAA (1.0), BAP (1.0)	Cormlet regeneration through somatic embryogenesis	
Shoots from lateral and apical buds	MS vitamins, Quorin and Lepoivre salts	BAP (5.0)	Shoot multiplication	Majourhat et al. (2006)
Small corms, apical buds attached to corm tissue	MS	BAP (6.0)	Multiple shoot induction	Sharma et al. (2008)
Small corms, apical buds attached to corm tissue	MS	IBA (3.0), BAP (14.0), Sucrose 5%	Multiple shoot induction	
Individual *in vitro* shoot	MS	IBA (3.0), BAP (14.0), Sucrose 5%	Shoot multiplication	
Shoot cluster (2-3 shoots)	MS	BAP (6.0), Sucrose 8%	Cormlet formation (1.84 per cluster)	
Shoot cluster	½ MS	BAP (3.0), Sucrose 8%	Cormlet formation (1.89 per cluster)	
In vitro cormlets	MS	BAP (12.0 or 14.0), IBA (3.0)	Apical and axillary buds sprouted	
Cold treated *in vitro* cormlets	MS	BAP (3.0)	Daughter cormlet formation	
Apical buds	LS	NAA (4.02), BAP (5.0)	Micro-corms regeneration	Mir et al. (2010)
Corm	B5	IBA (0.5)	Maximum root formation (76.9 %)	Sharifi & Ebrahimzadeh (2010)
	MS	IBA (4.0)	Maximum roots number (19.0)	
Lateral buds	MS	2,4-D (0.1), BAP (2.0), AA (100.0)	Shoot initiation	Zeybek et al. (2012)
Shoots	MS	BAP (1.0), AA (100.0)	Shoot growth	

Table 2.2, Continued.

	MS	IBA (1.0)	Corm formation	
	MS	IBA (2.0)	High roots number	
Corm sections	MS	2,4-D (0.25), BAP (1.0)	Callus initiation and growth	
Callus	MS	BAP (1.5)	Adventitious shoot initiation	
Shoots	MS	IBA (1.5), Sucrose 5%	Rooting and corm formation	
Corm slices	½ MS	BAP (4.5), NAA (3.72)	Highest callus formation	Quadri et al. (2010)
Callus	½ MS	BAP (4.5), NAA (3.72)	Mini cormlets formation	
In vitro cormelets	½ MS	BAP (0.45), NAA (0.37), Sucrose 6%	Notable size cormlet formation, multiple root regeneration and callus formation	
Lateral buds	½ MS	BAP (0.45), NAA (0.37), Sucrose 6%	No response	
Apical buds	½ MS	BAP (0.45), NAA (0.37), IBA (1.78), edible sugar 4%	Prominent size cormlets formation and maximum root formation	

2.3 Flowering in saffron

The reproductive stage or flowering is a unique developmental event and considered as a difficult process controlled by environment and physiological factors. The flowering process includes two developmental stage transitions: the transition from the juvenile vegetative stage to the mature adult vegetative stage and the transition from vegetative growth phase to reproductive growth phase followed by flower formation (Hong & Jackson, 2015). In saffron, the transition from the vegetative phase to flowering takes place shortly afterwards in the apical buds of underground corms, which do not have roots or foliage leaves. This transition has been reported to be different among different locations due to their differences in corm size and sessional variations. In Azerbaijan for instance, the reproductive stage of saffron starts during March (Azizbekova & Milyaeva, 1999), from March to April in Israel (Kumar et al., 2009), April to May in Spain (Lopez-Corcoles et al., 2015) and during July in Kashmir (Koul & Farooq, 1984). However, saffron life cycle in all growing countries is similar.

As mentioned, there are various environmental and agronomic factors that directly or indirectly control flowering and yield of saffron. Among the major factors include temperature, corm size/weight, fertilizer and manure, soil, irrigation, photoperiod, planting time, planting depth, plant spacing and plant density.

2.3.1 Effect of temperature

Temperature is one of the most important environmental factors controlling growth and flower emergence in saffron. According to some authors (Fernandez, 2004; Mollafilabi, 2004), rainy seasons, warm summers and mild winters are the optimal climatic conditions for saffron, even though some saffron are reported to show tolerance to low temperature of -18 °C (Mollafilabi, 2004), as well as high temperatures up to 40 °C (Gresta et al., 2009). Some others (Molina et al., 2004; Molina et al., 2005) suggest

that bud dormancy and floral initiation require high temperature, which is optimal in the range from 23 to 27 °C, with the former being more favorable for attaining maximum flowers. Therefore, the ideal temperature for flower formation of saffron should be markedly higher than flower emergence and vice versa (Molina et al., 2004).

Cold environment increases the number of flowers but decreases the amount of picrocrocin and crocin content which leads to lower quality of stigma (Gresta et al., 2009). Unusual low temperature and high humid conditions during short flowering period has a negative effect on flower formation. Flower initiation occurs in saffron during late spring, as the rising temperature reach above 20 °C, and as the temperature drops below 16 °C, flower starts to emerge (Kumar et al., 2009). Molina et al. (2004), quoted that maximum number of flowers were attained with corms incubated at 25 °C for more than 55 days prior to planting at 17 °C. The longer the duration of exposure, the shorter would be the time required for flower emergence and hence the longer the time needed from corm lifting to flowering. However, a long exposure to 25 °C exceeding 150 days showed a considerable decrease in flower number. They further stated that flower size was inversely related to the flowers produced per corm. In another experiment, Molina et al. (2005), quoted that incubation of saffron corms at a temperature other than 23-27 °C reduced number of flower per mother corm. Furthermore, the decrease was more noticeable at a higher temperature (30 °C) compared to lower temperature (21 °C), whereas the corms incubated at 9 °C failed to flower. Therefore, they further tested corms storage at temperature around 0 °C and found that freezing temperatures (0 °C and -1 °C) damaged the corms, whereas corms incubated at 1 °C and 2 °C produced more flowers. Overall, no beneficial effect of cold treatment on saffron flowering was demonstrated (Molina et al., 2005).

As mentioned, saffron requires high incubation temperature for flower differentiation followed by a low temperature period for flower emergence, Koocheki et al. (2011) claimed that corms incubated at 30 °C failed to flower, whereas longer incubation period adversely affected flower emergence of saffron. Moreover, the maximum number of vegetative buds and flowering response was attained with corms incubated for 90 days at 27 °C followed by forcing at 17 °C for flower emergence. It is proposed that increasing daily average temperature of saffron cultivated regions of Khorasan by 2 °C in summer and autumn season shows a remarkable delay in sprouting and flowering of saffron corms (Koocheki et al., 2011).

2.3.2 Effect of size/weight of mother corm

As productive phase is followed by vegetative phase during the growth phases of saffron, therefore, the size and weight of mother corms may have a significant impact on its yield and productivity. Saffron corm needs to be above a minimum size to produce flowers as flowering is directly linked to the corm size. Thus, cultivation of large sized corms gives more yield than smaller ones (Douglas et al., 2014). Moreover, the size of corm limits the presence or absence of flowers, given that if the corms do not attain their normal size, they only produce leaves (Renau-Morata et al., 2012).

Saffron forms flowers when the diameter of corm is above 1 cm (~1.1 g) (Benschop, 1993); however sometimes it takes 3 years for the progeny of 1 g mother corm to reach the certain mass to flower (Douglas et al., 2014). On the other hand, very big corms reduce the number of flowers per corm possibly due to aging, therefore, intermediate corms are suggested as the most suitable size to ensure optimal flower production (Mashayekhi et al., 2006). Commercially, mother corms which are 8-16 g in weight and 2.5-3.5 cm in dimension are highly recommended for saffron cultivation (Kumar et al., 2009) which is in agreement with the findings of Bayat et al. (2016) who found that using 10 g or bigger

corms of saffron increase flower number and fresh flower weight than using corms of 6 g or smaller corms.

In Spain, mother corms measuring 2.25-3 cm diameter yielded mores flowers than smaller ones (De Juan et al., 2009) and In Kargil, India, bigger corms (3.25-3.75 cm) gave the highest stigma length (4.93 cm), leaf length (47 cm), number of flowers per corm (2.45), and number of daughter corms (8.50) (Munshi et al., 2003). Likewise, in Italy, big mother corms improved the total weight of daughter corms by 16.6% (Gresta et al., 2008a) and in New Zealand, corms planted below 6.9 g did not flower (McGimpsey et al., 1997). In Saudi Arabia, the highest number of sprouts, leaves and progeny per corm were recorded when the mother corms weighing >10 g were planted; however, none of three corm weights (<5 g, ≥5–10≤ g, >10 g) produced saffron flowers, which might be attributed to the late cultivation of saffron corms in December (Sharaf-Eldin et al., 2013). According to Çavuş & Erkel (2009), daughter corms and stigma is not influenced by mother corm size after the first year if they are planted consecutively more than one year.

In another study, Douglas et al. (2014) investigated the production of 9 mother corm classes between 0.5-53 g and reported that bigger corms flowered 5-7 days earlier than the smaller corms, while very few flowers were produced with mother corms smaller than 2 g. They also found that mother corms >33 g produced more flowers (4, 16 and 48) than 11-15 g mother corms which produced 1, 4 and 20 flowers per original corm, whereas mother corm measuring 0.5 g did not produce any flower over the three generations.

Recently, Khorramdel et al. (2015) stated that there is a significant increase in the number and fresh weight of flowers per corm when bigger corms are used for planting. The author suggested that 5-10 g mother corms along with 15% concentration of foliar fertilizer is the most ideal combination to get highest number of flowers, fresh weight of flowers and dry weight of stigma. Hence, it is evident that corms with bigger size have a

higher physiological ability for early initiation of flowering and due to more food reserves, increase the percentage of flower emergence, and ultimately improve number of flowers and yield.

2.3.3 Effect of manure and fertilizer

Manure and fertilizers applications are the common amendments that are routinely used in agriculture for soil fertility and productivity. Manures generally improve soil physico-chemical properties by increasing pH in acid soils, hydraulic conductivity and infiltration rate, water-holding capacity of soil and reducing soil bulk density (Uzoma et al., 2011). In saffron, using manure alone does not meet the nutritional requirements, however the combination of Nitrogen, Phosphorus, and Potassium (NPK) along with organic manures increases flower yield and quality. The optimum availability of nutrients, especially N and P, is suggested to be one of the most important factors affecting flower and saffron yield (Koocheki et al., 2014).

In Kashmir, cultivation of saffron without the supply of nutrients to the fields has considerably decreased the fertility of the soil. The application of fertilizers and organic manures is therefore recommended to enrich the soil with an adequate quantity of essential nutrients and to improve the yield of saffron (Kirmani et al., 2014). Results from Budgam District of Kashmir Himalayas, India, demonstrated that the highest saffron yield of 3.64 and 3.51 kg ha^{-1} were obtained when N and farm yard manure (FYM) applied at 90 kg ha^{-1} and 60 ton ha^{-1}, respectively (Kirmani et al., 2014). A study from Iran demonstrated that 15% concentration of Dalfard 15® foliar fertilizer along with 5-10 g mother corms weight were the most ideal condition for the whole vegetative and reproductive traits of saffron (Khorramdel et al., 2015). Likewise, Hosseini et al. (2004) stated that the application of foliar fertilizer increased the number of flowers by 33% during the month of March. On the other hand, Alidadi et al. (2013) examined the effect

of bio-fertilizers such as compost, vermicompost and sulfur compost on saffron yield and observed that use of 8 kg of each fertilizer compared with 16 kg increased saffron yield by 19%. Among the fertilizers used, sulfurous granular compost was highly effective (4 ton ha^{-1}) to saffron yield (Alidadi et al., 2013).

Besides manure and fertilizers, recent research on nano fertilizers in a number of crops has revolutionized conventional agricultural system indicating their potential use in the improvement of crops. Some studies highlighted the importance of nano fertilizers in saffron yield improvement. In this way, Azarpour et al. (2013) demonstrated that foliar spraying of nano iron fertilizer had significant effect at 1% probability level on fresh flower cover yield of saffron. In another study, all nano fertilizers (especially Fe) significantly increased the production of entire vegetative and reproductive traits (Amirnia et al., 2014). In general, it can be concluded that the flowering and yield of saffron are strongly influenced by farm management such as cattle manure, particularly cow manure compost.

2.3.4 Soil requirement for saffron

Soil quality is widely considered to be one of the key parameters maintaining sustainable land use management and attaining maximum yield. Understanding and assessing the influence of land use and management on soil quality are two important goals for modern soil science, which can play a major role in the conservation and improvement of soil quality and crop production (Spurgeon et al., 2013). Although, there is a growing emphasis on increasing the productivity of crops, fruits and herbs using efficient application of agricultural inputs without degrading the soil or environment, however little research is available on practices related to soil management in bulbous plants including saffron.

Regarding the relationship between soil quality and cultivation, saffron is adapted to grow on a wide range of soils ranging from sandy loam to clay loam but grows well in low dense, friable, well-drained and well-watered clay-calcareous soils with a fairly loose texture and suitable permeability (Kumar et al., 2009). Unlike, majority of the plants that prefer slightly acidic to neutral soil, the optimum pH of the soil for saffron lies between neutral to slightly alkaline, with the latter being highly recommended for giving better stand of the plant (Gresta et al., 2008b). Heavy clay and poorly drained soil affect saffron yield as water from rainfall or sprinklers collected on the surface of the soil cause rotting of corms.

In New Zealand, well-drained soil with a sandy or loamy texture is suggested as the best soil for saffron production (Deo, 2003). Likewise, the saffron growing soils of Kashmir has high colour value, more alkaline and significantly higher number of alkaline earth carbonates in comparison to adjacent non-saffron growing fields (Kumar et al., 2009). In addition, the soils are calcareous in nature having a mean calcium carbonate ($CaCO_3$) and organic carbon content of 4.61% and 0.35%, respectively (Ganai et al., 2000). Soil enriched in $CaCO_3$ is preferred for optimal growth of saffron, as $CaCO_3$ facilitates the availability of trace elements for the crop and reduces toxic heavy metals in soil (Zeng et al., 2016).

Gresta et al. (2010) studied the effect of soil texture on saffron production in Catania plain, South Italy, and reported that sandy and sandy-silty soil resulted in higher flower and stigma yield while clay soil seems unsuitable for good saffron production. According to Tammaro (1999), Navelli area of Central Italy possesses a medium humus-clay soil, which assures good water storage for saffron production. Furthermore, saffron is grown on loamy sandy soil in Azerbaijan (Azizbekova & Milyaeva, 1999) and either loamy sandy or calcareous clay soil having a quite loose texture in Morocco, with the latter type

being more in the regions of Zagmouzen, Taliouine and Agadir Melloul (Ait-Oubahou & El-Otmani, 1999; Tahri et al., 2015).

2.3.5 Effect of irrigation

Water is becoming the critical limiting factor for the agricultural system, especially in arid and semi-arid regions. Therefore, low water requiring crops including saffron are cultivated in such zones to increase the return from water and land. Saffron is not even irrigated in many cultivated areas in the Mediterranean climate of Sardinia, Abruzzo, Spain and Greece (Tammaro, 1999). In Kashmir, saffron is cultivated under rain-fed environment (1000–1500 mm annum^{-1}) due to the unavailability of water resources. Therefore, farmers are highly dependent on mid-September rains for optimum growth of corms and facilitation of flowering. In this context, rainfall between 100 and 150 mm is considered crucial immediately before flowering of saffron, as moisture in the soil stimulates flower initiation and emergence. Thus, light irrigation during late August and September helps to boost blooming and enhance the production of this crop (Kumar et al., 2009). However, over the last several years, change in climate has adversely affected flowering and yield in saffron as the weather has become more erratic and rainfalls are quite scanty. For example, Kashmir was affected by an acute drought during 1999 to 2003, due to which the production of saffron reduced from 3.12 to 1.57 kg ha^{-1} (Husaini et al., 2010). Therefore, an appropriate irrigation method is required for achieving high yield of saffron in Kashmir.

The use of proper irrigation strategies plays a vital role in farm management. Furthermore, using a better irrigation strategy improve the growth and total yield of the crop. As mentioned, saffron growth in arid and semi-arid conditions is favored, therefore, the main water source for saffron irrigation in the arid and semi-arid areas is well water. However, due to problems like soil salinization and methane seeping, more efficient use

of this resource is needed. For such a purpose, some authors have highlighted the need for suitable irrigation practices of saffron in order to provide enough water to fulfill the evaporative demand and reduce salt accumulation in the soil (Sepaskhah & Kamgar-Haghighi, 2012; Yarami & Sepaskhah, 2016). Sepaskhah & Yarami (2009) for instance, evaluated the interactive effects of different levels of saline water and irrigation on saffron growth and flower yield. The results illustrated that among all the growth components of saffron, flower and corm productivity are the most and least sensitive to water stress and salinity, respectively. The authors claimed that when saline water is used for irrigation of saffron, the irrigation interval should be more frequent (2-d interval) to overcome severe water stress (Sepaskhah & Yarami, 2009).

Traditionally, saffron is irrigated through basin irrigation method in Iran. However, Azizi-Zohan et al. (2009) postulated that using furrow irrigation as an alternative irrigation method for saffron results in a decrease of evaporation surface and increase in water use efficiency. Therefore, the effects of rainfall, irrigation methods as well as irrigation intervals on saffron production under semi-arid condition were tested. Their finding showed that saffron yield with basin irrigation method (2.32 and 5.49 kg ha^{-1}) was higher than furrow irrigation (0.53 and 1.20 kg ha^{-1}) in 1998-1999 and 1999-2000, respectively. Furthermore, in 2000, basin irrigation resulted in a mean production of 10.8 ton ha^{-1} corm, which was significantly higher than corm produced in furrow irrigation (6.5 ton ha^{-1}). Additionally, saffron production using basin and furrow irrigation method gave the highest and lowest water use efficiencies of 1.86 and 0.24 g m^{-3}, respectively, with 24-day intervals in 2000. This contrasts with the findings given by Yarami & Sepaskhah (2015), who examined the impact of irrigation water salinity and planting method on yield and growth of saffron under field conditions in Iran, and reported that in-furrow planting method resulted in more than 3.5 times higher saffron yield compared with basin planting, showing that the in-furrow planting method is an appropriate method

for saffron production, probably due to ideal soil temperature condition for corms growth. Thus, an in-depth knowledge regarding suitable irrigation method under arid and semi-arid regions could help to enhance the growth and yield of saffron.

2.3.6 Effect of planting time

Planting at the most appropriate time has a remarkable influence on the growth and yield of crops. Once the best planting period of a crop has been determined, any delay in sowing at that period may negatively affect the qualitative and quantitative characteristics of the crop. As with many traditional crops, the planting time of saffron varies in different parts of the world, depending upon climatic conditions. In Spain, for example, the suitable time to plant saffron corms is second half of June, from the second half of August in central Italy, before the mid of September in Greece, from the mid of July to the end of August in India (Negbi, 1999), from July to August in Azerbaijan (Azizbekova & Milyaeva, 1999) and from late August to September in Morocco (Ait-Oubahou & El-Otmani, 1999).

Saffron is mainly planted between late July and late September in Iran (Khorasan), however due to factors like plant physiology, development processes, and organs differentiation in saffron corm, early June is recommended for higher yield of saffron (Mollafilabi, 2004). Rehman & Lodhi (1977) observed that in Balochistan province of Pakistan, the highest corm yield was obtained from corms planted in the middle of July followed by intermediate and lowest yield from June and August plantings, respectively. In Mashhad region of Iran, Amirnia et al. (2013) studied the influence of planting date on stigma yield and yield components in saffron and concluded that 05-June and 05-July were the best dates for planting corms in Mashhad, due to their positive effects on yield as compared to sowing dates of 05-May, 05-August, 05-September, and 05-October. In addition, delaying in sowing time not only reduced yield and yield component of saffron

for the current year, but also affected its yield in the following years. In central Sicily, Italy, an earlier planting time (end of July) resulted in greater number of flowers, stigma yield as well as total yield in comparison to later one (end of August). Moreover, early planting time gave a significantly higher quality stigma, suggesting that planting of saffron at an optimum time play a crucial role in the accumulation of secondary metabolites (apocarotenoids) responsible for stigma quality (Gresta et al., 2008a).

2.3.7 Effect of planting depth

Planting of saffron corms at optimum depth is hypothesized to influence saffron yield though Negbi et al. (1989) pointed out that the agricultural factors are not affected by planting depth. The contractile roots of saffron become thick and fleshy when mother corms are cultivated to less than 8-10 cm, making them unable to produce daughter corms. It is because the reserve material is stored in contractile roots and the mother corm is wholly consumed by them (Negbi et al., 1989).

Depending upon soil texture, the recommended sowing depth for saffron corms varies from 7.5-10 to 15-22 cm in different regions. The planting depth in Iran (15-20 cm) is much higher than Kashmir (10-15 cm) (Kafi & Showket, 2007). Therefore a study carried out in Iran stated that increasing planting depths from 10-15 cm increase flower number and corm weight of saffron but decrease flower emergence (Galavi et al., 2008). It shows that increasing the planting depth decreases the corm bud temperature site that results in breaking of bud dormancy and thereupon enhances flowering time. In India, corms planted in 7.5 and 10 cm accumulated highest dry matter than corms planted in 12.5 and 15 cm in initial stages but the trend was reversed in later stages (Kumar et al., 2012), whereas De Juan et al. (2009) planted saffron at a depth of 10 and 20 cm in Albacete, Spain, and reported highest number of flowers m^{-2} and per planted corm by planting saffron at 10 cm depth. However, planting depth of 20 cm resulted in the highest saffron

qualitative production (fresh weight of stigmas per flower) than 10 cm and this could be the reason why farmers usually sow their corms relatively deep (De Juan et al., 2009).

2.3.8 Effect of plant density

The response of flowering and yield to plant density has been widely studied in saffron. De Juan et al. (2009) reported that saffron yield was affected by plant density in contrasting ways under warm Mediterranean region of Spain. At high plant density (69 corms m^{-2}), an increase in yield per unit of surface was observed, whereas at low plant density (51 corms m^{-2}), yield increased with respect to the corms initially planted. In another experiment at Albacete, Spain, big sized saffron corms grown at 200 and 300 m^{-2} recorded maximum corm yield of 28.4 and 36.3 ton ha^{-1}, respectively (De Juan et al., 2003).

While comparing 4 planting densities (50, 100, 200 and 300 corm m^{-2}) in Iran, Koocheki et al. (2014) found highest flower and corm yield at planting density of 300 and 200 corms m^{-2} in the first year and second year, respectively. The authors suggested crop density of 200 corm m^{-2} for saffron in Iran. Similar results were obtained by Koocheki et al. (2016). In central Silicy, South Italy, plant density positively affected flower number per unit area but negatively affected unitary stigmas weight (Gresta et al., 2009) therefore medium plant density (111-119 corms m^{-2}) of saffron corms was recommended by Temperini et al. (2009) for farmers due to its similar spice yield and maximum daughter corms production than high density treatments.

2.3.9 Effect of plant spacing

Spacing is a parameter used by farmers so as the adjoining corms are planted at a uniform distance apart. Like all other cultural management elements, plant spacing shows large and predominant influences on growth, development, and yield of saffron. Narrow plant spacing impedes intercultural operations and enhances competition among the

plants for nutrients, light, and air, resulting in weaker and thinner plants and consequently, reduces the yield. In addition, more corms and more labour work are needed for planting saffron corms under narrow spacing, which is a losing concern. On the other hand, wider plant spacing increases the competition among weeds and crop plants, and reduces plant stand, thus reduce the yield per unit area. Therefore, optimum plant spacing must be adapted to ensure maximum yield of saffron.

As mentioned, plant spacing is among the key productivity factors and various studies are carried out in saffron to determine its effect on flowering and yield. Under mid hill sub-temperate environment in Palampur, India, Kumar et al. (2012) recorded 51.8% higher corm weight and 19.2% higher corm diameter with wider spacing (15x10 cm) than narrow spacing (10x7.5 cm) but no significant variation in corm yield was observed. In Iran, corms planted in four different plant distances (5x20, 10x20, 15x20 and 20x20 cm) were non-significant in terms of fresh and dry matter stigma yield as well as fresh flower yield. However, an increase in plant distance decreased fresh and dry matter stigma yield and fresh flower yield (Mohammad-Abadi et al., 2007). In an attempt to grow saffron in semi-arid Bekaa valley of Lebanon, a far spacing of 20x20 cm gave bigger corms, higher flower number and stigma yield as compared to 20x10 cm and 10x10 cm spacing (Yau & Nimah, 2004). In Italy, planting saffron corms at a spacing of 2-3 cm in furrows gave the highest yield of flower and corms (Skrubis, 1990). Likewise, corms are sowed in furrows made with a plough at a spacing of 25x12 cm in Greece (Goliaris, 1999).

2.3.10 Effect of photoperiod

Photoperiod is one of the main environmental signals controlling flower initiation and formation in plants. However, little is known about the growth and flowering response of saffron to photoperiod. Dhar (1990) marked an optimum period of 11 h illumination for flower formation in saffron. Koocheki et al. (2007) reported that 33% of corms initiated

flower located under 16/8 (light/dark) photoperiod compared with 75% for corms placed under natural conditions. However, none of the corms grown under light regimes of 6.5/5.5-initiated flower.

2.4 Gamma irradiation

Mutation breeding is one of the most frequent techniques to develop additional variability in crop plants including vegetatively propagated flowering plants. The main advantage of mutation breeding in seed and vegetatively propagated crops is the ability to change plant characteristics for a significant increase in plant production without altering the basic genetic makeup of the genotype (Jain, 2005). Mutagens such as chemical (colchicine and ethylmethanesulphonate), ethyl-methane-sulphonate (EMS), sodium azide (SA), methyl-nitroso-urea (MNH), ethyl-nitrosourea (ENH), and physical agents (gamma rays, X-rays, neutrons, UV, and laser) are commonly used as a mutagen (Jain et al., 2010). Among these mutagens, gamma rays, chemicals and EMS are most frequently used to induce mutation (Amir et al., 2018).

Physical mutagens have accurate dosimetry and reasonable reproducibility, and high and uniform penetration of multicellular system, particularly by gamma rays, whereas the advantage of using chemical mutagens is a high mutation frequency and predominantly point mutations (Jain, 2005). Mutation induced by chemicals or physical agents have high efficiency to generate mutation in various crops and ornamental plants. Amongst all these mutagens, radiation (64% with gamma rays and 22% with X-rays) is the most common method to induce mutation in plants (Jain, 2005). The irradiation method play a vital role in irradiation mutagenesis (Ru-Fang et al., 2005), flower antistaling (Hayashi & Todoriki, 1996), plant physiology (Jun et al., 2006), farm product storage (Lao & Fu, 2004), and species breeding and selection (Chen & Zhao, 2004). Furthermore, gamma rays have improved the characters and productivity of many crops of our daily diet such as cowpea,

bean, rice, maize and potato and is still showing a higher potential for improving vegetatively propagated plants (Asare et al., 2017).

2.4.1 Gamma radiation in saffron

Khan (2004) exposed saffron corms with gamma rays at different doses ranging from 0.5 krd to 3.0 krad using Cobalt 60 source and observed that the number of sprouted bulbs and percentage of plants surviving reduced with increase in irradiation dose. Maximum corm yield and plant height was showed by corms irradiated with 0.5 krad followed by a significant decrease with increase in irradiation. Delayed sprouting and slow growth in higher doses from 2.0-3.0 krad and flower induction in middle doses from 1.0-2.0 krad was also observed.

In an attempt to determinate the effect of gamma radiation on saffron flowering and development, saffron corms weighing between 12-14 g were exposed to various doses of gamma rays (0, 5, 10, 15, 20 and 25 Gy) with ^{60}Co (Jun et al., 2006). The results indicated that Gamma rays inhibited leaf and style growth with increase in dose when compared to control plants. On the other hand, higher doses (10-25 Gy) showed maximum flowering ratio (108.6%) compared to the control plants (106.7%). Furthermore, there was no significant difference in blooming time of corms treated with 5 Gy dose and the control. However, corms exposed to 10-25 Gy delayed the blooming time (3 to 6 days) compared to control plants. In addition, young plants raised from 20 and 25 Gy irradiated corms were small in height with zero survival rate while plants grown from control, 5 and 10 Gy irradiated corms showed survival rate of 90%, 34%, and 31%, respectively. Totaling, gamma rays of 5 and 10 Gy were the most favorable doses for the entire vegetative and reproductive traits of saffron corms (Jun et al., 2006).

In another study, saffron corms were irradiated with gamma rays at 0.25-1 kr doses with a ^{60}Co source. Higher dose (1 kr) delayed sprouting response of irradiated corms.

Moreover, reduction in corm yield and dry pistil weight was also observed in higher dose due to strong decrease in number of flowers and/or percentage of flowering plants (Nehvi et al., 2007).

Nehvi et al. (2010) investigated the effect of physical and chemical mutagens on variability for floral, morphological, and anatomical traits in saffron and found that among all the doses of gamma rays (0.1, 0.2, 0.3, 0.4, and 0.5 krad) 0.2 krad showed maximum corm sprouting percentage (>99%) in M1 generation followed by a significant decrease with increase in dose and recorded minimum (68.0%) with radiation dose of 05 kr. Furthermore, in M1 and M2 generations, maximum number of flowers per corm (2.31), maximum flower weight (0.589 g) and maximum fresh pistil weight (0.084 g) were exhibited by 0.2 kr radiation.

2.5 Toxicity

Toxicity is the scientific study of the undesirable effects of bioactive substances on living systems. Toxicological screening is very important to establish the safety and efficiency of any new drug and to determine if the testing drug can be approved for clinical use. Depending upon the duration of animal exposure to chemical, toxicological studies are of four types: acute, sub-acute, sub-chronic and chronic studies.

2.5.1 Acute toxicity

It is the undesirable reactions occurred immediately as a result of the administration of a single dose of the chemical, or multiple doses given within 24 hr (Duffus et al., 2009). This type of assays is conducted on either mice or rats because of being economical and easily available. Moreover, the toxicological reference data for many compounds are available in these species. Also, these animals are considered to have a similar metabolism manner and metabolites pharmacodynamics to human (Martignoni et al., 2006). Acute toxicity tests are commonly designed to evaluate the Lethal Dose 50 (LD_{50}) of the tested

substance. Statistically, LD_{50} is the amount of a chemical that can be expected to cause the death of one-half of the experimental animals exposed to it.

2.5.2 Sub-acute toxicity

In sub-acute toxicity test, repeated doses of drug is administered in sub-lethal amount for a treatment period of 14 to 28 days (Colerangle, 2017). Sub-acute toxicity studies are conducted to determine the effect of drug on biochemical and hematological parameters of blood as well as the histopathological changes (Haque & Haque, 2011). Furthermore, it is performed as range-finding studies in order to select dosage levels of drug to be used in subsequent sub-chronic and chronic toxicity studies (Colerangle, 2017).

Sub-chronic toxicity is the term used to describe the negative consequences of repeatedly exposing experimental animals to a toxin on daily basis. Drugs are administered in various doses for a 90-day period in sub-chronic toxicology investigations to ascertain their potential for mutagenicity and carcinogenesis (Parasuraman, 2011). The requirements for research on sub-chronic toxicity are the same as those for research on sub-acute toxicity. Research involving many doses are required to confirm the health benefits of herbal remedies.

2.5.3 Sub-chronic toxicity

Sub-chronic toxicity is the term used to describe the lethal effects occurred due to the repeated daily exposure of experimental animals to a drug. Normally, drug is given in different doses for a period of 90 days in sub-chronic toxicity studies to evaluate their potential for carcinogenesis and mutagenicity (Parasuraman, 2011). The parameters of sub-chronic toxicity studies are similar to that of sub-acute study. Multiple dose studies are required to assure the safety of herbal remedies.

2.5.4 Chronic toxicity

Chronic toxicity study can only be performed for test doses that passed the subchronic toxicity test and starts 24 hours after the last sub-chronic dose was administered (Chinedu et al., 2015). The test compound is administered over more than 90 days to a year (Parasuraman, 2011). Like shorter repeated dose toxicity study designs, the purpose of chronic toxicity study is to further test the hypothesis regarding mode of action, detect target organs, predict the health consequences of therapeutic entity in human exposure, further characterize the dose-response relationship, and determine a dose range that does not show lethal effects with chronic use (Krewski et al., 2010).

This phrase describes the process by which substances or medications are given to individuals or animals. There are a number of ways to administer a substance in order to test its toxicity in livestock, but the two most popular methods are intraperitoneal injection and oral administration.

2.6 Routes of administration

This term describes the way in which chemicals or drugs are given to humans or animals. There are various routes used to evaluate toxicity of a compound in animals, but the two most commonly used routes of administration are: i) intra-peritoneal injection, and ii) the oral route.

2.6.1 Intra-peritoneal injection

Intra-peritoneal or IP injection is the injection of a pharmacological drug into the peritoneal cavity. Intraperitoneal injection is widely used in rodents as a route of drug administration. IP technique is quick and minimally stressful for animals. It involves holding of the rodent in a supine position with its head tilted lower than the posterior part of the body and insertion of the needle in the lower quadrant of the abdomen at an angle of ~10° with care to ensure minimal danger of perforation of abdominal viscera (Simmons

& Brick, 1970). Up to 10 ml/kg of the solutions can be safely administered to rodents through IP which may be advantageous for agents with poor solubility. This route is commonly used in chronic studies involving mice for which repetitive IV access is challenging. IP route, in most cases, is also preferred over the oral route for biological agents in order to avoid the gastrointestinal tract and potential modification of biopharmaceuticals (Al Shoyaib et al., 2020).

2.6.2 Oral administration

The oral route is probably convenient, cost-effective, and one of the most common forms of drug administration. In short, oral administration refers to a method of administration that involve the gastrointestinal tract, which may be viewed as a tube going through the body from the mouth to the anus. Although it is within the body, its components are basically outside the body fluids. Most orally administered chemicals can otherwise have a systemic effect on the organism only after absorption has occurred from the mouth or the gastrointestinal tract. Oral administration of chemicals that are rapidly absorbed from the gastrointestinal tract would theoretically expose the liver to concentrations of the agent that would not be obtained if other routes of administration were used (Loomis and Hayes, 1996). However, most drugs are usually absorbed from the small intestine. The drug passes through the intestinal wall and travels to the liver before being transported via the bloodstream to its target site. The intestinal wall and liver chemically alter (metabolize) many drugs, decreasing the amount of drug reaching the bloodstream (Murakami, 2017). That is why these drugs are administered in relatively smaller doses when injected intravenously to achieve the desired effect.

2.7 Chemistry of Saffron

Saffron contains a multitude of phytochemical ingredients associated with various classes of natural substances, based upon the origin, processing conditions and storage

period. The most significant constituents involved in eliciting pharmacological and pharmaceutical activities are described below.

Based upon the extensive chemical analysis, it was revealed that the saffron stigmas contain more than 150 phytochemical ingredients, belonging to various divisions of secondary metabolites such as carotenoids, flavonoids, terpenoids and anthocyanin (Bathaie & Mousavi, 2010). Amongst them, carotenoids emerge as the major constituents of saffron stigmas, primarily responsible for imparting a distinct colorant and aroma to the spice (Gismondi et al., 2012). Studies have reported the prevalence of both lipophilic and hydrophilic carotenoid contents within saffron. Where the lipophilic carotenoids, comprising alpha-carotene, beta-carotene, phytoene, phytofluene, zeaxanthin, and lycopene are found in minor amounts; the hydrophilic carotenoids demonstrate relatively increased levels, which include crocetin, its glycosidic forms digentiobioside (crocin), glucoside and gentiobioside (Fernandez, 2004; Pfander & Schurtenberger, 1982).

Crocetin is a polyene dicarboxylic acid that makes dark red crystals having a melting point of 285 °C (Bolhasani et al., 2005). Where only 6% of crocetin is present in free form within saffron, the remaining 94% is present in glycosidic form (Moghaddasi, 2010).

The intense orange-red colour of saffron stigmas is primarily due to the presence of crocetin glycosyl ester, crocin (C_{44} H_{64} O_{24}) with the IUPAC name as 8, 8-diapo-8, 8-carotenoic acid (Srivastava et al., 2010). It constitutes between 6-16% of total dry mass of saffron. The structure of crocin, was reported by Karrer and Kuhn during the period 1927–1931 (Karrer et al., 1928; Karrer & Miki, 1929; Karrer & Salomon, 1927, 1928a, 1928b). Bearing a striking deep red colour, it tends to develops crystals with a melting point of 186 °C (Bolhasani et al., 2005). Apart from many species of genus *Crocus*, Crocin is also present in *Jacquinia angustifolia* (Eugster et al., 1969), *Coleus forskohlii* (Tandon et al., 1979), *Buddleja officinalis* (Liao et al., 1999), *Nyctanthes arbor-tristis*

(Gadgoli & Shelke, 2010), the fruit and flower of *Gardenia jasminoides* (Pfister et al., 1996), and *Artocarpus heterophyllus* (Priyadarshani et al., 2009). Other carotenoids like β-crocetin, γ-crocetin and mangicrocin have also been reported in saffron stigma (Kumar et al., 2009). Figure 2.4 shows the chemical structures of main saffron constituents.

The characteristic bitter flavor and aroma of saffron are mainly due to the carotenoid oxidation products- picrocrocin and its deglycosylated derivate safranal, respectively. Picrocrocin ($C_6H_{26}O_7$), a colourless monoterpene aldehyde, is a degradation product of the zeaxanthin carotenoid responsible for the distinctive bitter taste of saffron (Srivastava et al., 2010; Yasmin & Nehvi, 2013). According to Carmona & Alonso (2004), other compounds, such as flavonoids, also contribute their role in giving saffron a bitter flavor. The solubility of picrocrocin in water is more than in water-alcohol solutions but totally insoluble in apolar ones (Alonso et al., 2001). Picrocrocin, the second most rich constituent by weight, represents 1-13% of dry matter of saffron (Sobolev et al., 2014). Alonso et al. (2001) observed the carotenoid contents of saffron from Iran, India and Spain and found their picrocrocin content as 2.18-6.15% (Iranian), 1.07-2.16% (Indian) and 0.79-12.94% (Spanish), respectively.

The natural de-glycosylation of picrocrocin induces the formation of the volatile aromatic aldehyde, safranal ($C_{10}H_{14}O$), which makes up to 70% of the total volatile compounds within the plant. (Gresta et al., 2008b; Husaini et al., 2010). For years, safranal was considered to be the sole aroma inducing compound in saffron; however, later studies revealed the prevalence of additional volatile constituents playing a pivotal role in generating saffron's distinct aroma (Straubinger et al., 1998; Tarantilis & Polissiou, 1997). These compounds include 4-ketoisophorone, 2,6,6-trimethyl-1,4-cyclohexadiene-1-carboxaldehyde, isophorone, 2-hydroxy-4,4,6-trimethyl-2,5-

cyclohexadiene-1-one and 2,2,6-trimethyl-1,4-cyclohexanedione (Carmona et al., 2007; Melnyk et al., 2010).

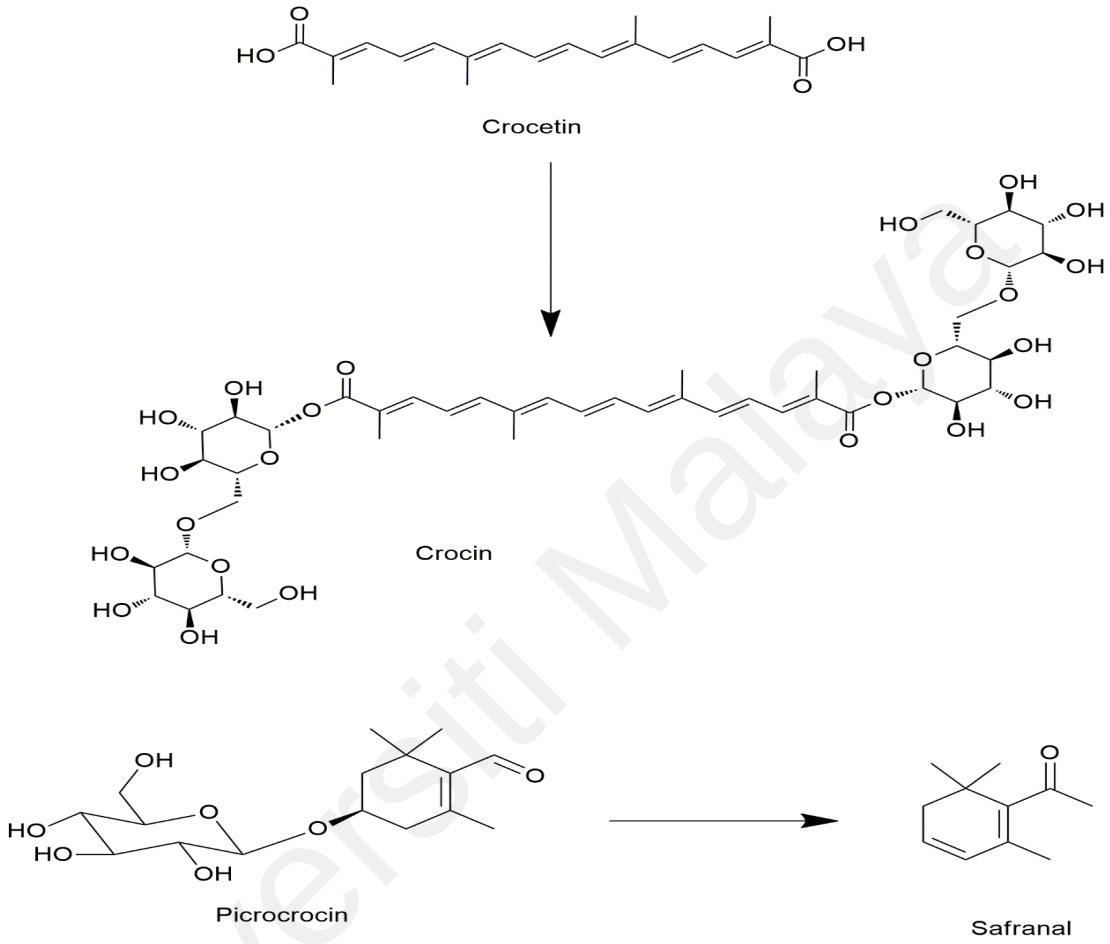


Figure 2.4: Chemical structures of main constituents of *C. sativus*

Other minor constituents such as terpenoids, flavonoids, anthraquinones, and anthocyanin have also been extracted from stigmas and other parts of saffron plants mainly petals, pollens and corms (Gresta et al., 2008b). Terpenoids such as crocusatins, found in stigmas and petals of saffron, possess significant antityrosinase activity and are amongst the most recovered constituents (Jan et al., 2014). Several glycosidic derivatives of terpenoids present in the saffron are the precursors of volatile saffron elements alternative to picrocrocin. Moreover, a series of flavonoids (all glycosidic derivatives of kaempferol) have also been characterized in the stigma and petals of saffron which, together with picrocrocin, give the characteristic bitter feature to this spice (Carmona & Alonso, 2004; Hadizadeh et al., 2010). In addition, some anthraquinones (Gao et al., 1998) and anthocyanin (Goupy et al., 2013) secondary components are also reported to be extracted from corms and petals of saffron.

2.8 Pharmacological activities of Saffron

Saffron and its active components such as safranal, crocin, picrocrocin, and crocetin have been reputed to be efficacious for the treatment of many disorders. Some non-volatile compounds, of which most of them are carotenoids such as lycopene, alpha, and beta carotene and zeaxanthin also play their role in pharmacological activities of this golden spice.

2.8.1 Antibacterial effect

Human infectious diseases caused by pathogenic bacteria is considered one of the major causes of morbidity and mortality, particularly following the emergence and spread of antibiotic resistant bacterial strains which have increased the risk of treatment failure (Li & Webster, 2018). However, some medicinal plants including saffron can be used as alternative antibacterial agents with therapeutic potential against antibiotic-resistant pathogens (Chen et al., 2017; Moghaddam, 2010). The antibacterial action of saffron

callus and stigma showed varying inhibitory effects on pathogenic bacterial strains. Saffron stigma extract inhibited *Staphylococcus aureus* at the lowest concentration (200 $\mu g.ml^{-1}$) compared to saffron callus (300 $\mu g.ml^{-1}$) (Parray et al., 2015). De Monte et al. (2015) examined the antibacterial efficacy of crocin, safranal and synthesised semi-synthetic derivatives of safranal from *C. sativus* against *H. pylori*. The MIC_{50} value of safranal against *H. pylori* strains was 32 μg/ml, while some of the semi-synthetic derivatives of safranal showed stronger antibacterial activities compared to the reference drugs clarithromycin (≥32 μg/mL) and metronidazole (>32 μg/mL). Crocin and safranal have been reported for their bactericidal effect in food contamination caused by *Salmonella enterica* (Pintado et al., 2011). Saffron petal being an effective antibacterial agent against *E. coli, P. aeruginosa,* and *S. aureus* can be used as a natural dye in clothing and textile industries (Ghaheh et al., 2014). Furthermore, saffron petal has potential application in food system as a natural preservative (Gandomi Nasrabadi et al., 2012).

2.8.2 Antifungal effect

Several reports devoted to antifungal activities of saffron and its constituents are available in the literature. According to Carradori et al. (2016), *Candida* spp. were shown to be highly sensitive to crocin, safranal and one of safranal derivative, thiosemicarbazone with concentration in the range of 0.0078–8 mg/ml. In another experiment, the lyophilized internal and external parts of saffron corms showed antifungal activities against *Rhizopus nigricans, Penicillium raistrickii, Fusarium oxysporum, Bipolaris spicifera* and *Aspergillus niger* (Rubio-Moraga et al., 2011). The authors suggested that more strong effect of saffron corm peel might be due to the presence of triterpenoid saponins azafrine 1 and azafrine 2 (Rubio-Moraga et al., 2013). Another study showed that the MIC value of petroleum ether and methanolic extracts of saffron stigma against fungal strains ranged from 2.13-3.2 mg/ml and 3.13–3.2 mg/ml, respectively. Moreover, the petroleum ether extract showed most effective MIC value for *C. albicans* and methanolic extract

for *Aspergillus fumigates*. The MIC values were significantly higher than the reference drug, amphotericin-B (Muzaffar et al., 2016).

2.8.3 Antioxidant effect

C. sativus stigma containing crocin, safranal, crocetin and phenolic compounds possess effective intracellular free radical scavenging effect and protect cells and tissues from oxidative damage. Serrano-Díaz et al. (2013) reported that among all the tested saffron parts, stamens represented the most potent hydroxyl OH(•) and peroxyl LOO(•) radicals scavenging property, more higher than those of food antioxidant, propyl gallate. According to Sánchez-Vioque et al. (2012) *C. sativus* leaves showed the best antioxidant properties by totally inhibiting β-carotene oxidation at 10 μg/ml and exhibited a DPPH scavenging property up to 32 times higher than those reported for grapes and berries, traditional sources of antioxidants. Likewise, tepal extract extensively inhibited oxidation of β-carotene, and showed scavenging NO radical and Cu^{2+} chelating properties. In another experimental research, the methanolic extract of *C. sativus* stigmas depicted strong radical scavenging activities, while callus extracts had maximum inhibition of peroxy-radicals in lipid peroxidation assay. The promising antioxidant activity of *C. sativus* callus extracts attributes to the presence and synergistic effects of similar bioactive compounds present in the saffron plant such as safranal, picrocrocin and crocin (Parray et al., 2015). Therefore, researchers are now paying more attention to various other by-products of saffron, such as stamens, spathes perianth, tunics, corms and leaves due to their antioxidant and free radical scavenging properties (Baba et al., 2015; Lahmass et al., 2018; Montoro et al., 2012; Smolskaite et al., 2011).

2.8.4 Analgesic effect

During the last decade, researchers from several laboratories have reported that traditional herbal medicine, including saffron have analgesic effect (Amin et al., 2017;

Bartels et al., 2006; Safakhah et al., 2016). Acute administration of saffron stigma and safranal extracts has shown potent analgesic efficacy in several models of analgesic assays in rodents such as xylene-induced ear edema, hot plate, the acetic acid visceral nociception test (Amin & Hosseinzadeh, 2015). Meanwhile, crocin (0.1 and 0.2 g/kg) showed significant anti-edematogenic potential in histamine-induced paw edema in rats. In a combined treatment of crocin (0.1 g/kg) with chlorpheniramine (2.5 mg/kg) a more documented response was observed in comparison with crocin (0.1 g/kg) and chlorpheniramine (2.5 mg/kg) treated alone (Tamaddonfard et al., 2012). In a subsequent study, it indicated that crocin (1 g/kg) increases morphine-induced antinociception; however, naloxan failed to reverse this action (Tamaddonfard & Hamzeh-Gooshchi, 2010). However, according to Hosseinzadeh & Younesi (2002), aqueous and ethanolic stigma and petal extracts of saffron at any dose exerted no significant analgesic effect in mice and suggested that the extracts might not act through central mechanisms, although drugs that alter the animals motor ability may enhance the licking duration on the hot plate method without acting on the central nervous system.

2.8.5 Anti-inflammatory effect

Phytochemicals screening of stigma and petals aqueous and ethanol extracts on acute inflammation by xylene-induced ear edema indicated that only stigma aqueous and ethanol extract at higher dose possessed anti-inflammatory effects in mice. However, stigma aqueous and ethanol extract as well as petal ethanol extract showed significant activity against chronic inflammation using formalin-induced edema in rat paw (Hosseinzadeh & Younesi, 2002). In another study, saffron aqueous extract suppressed formalin-induced paw edema in the chronic inflammation but failed to show activity against acute phase of a formalin test (Arbabian et al., 2009). Similarly, intraperitoneal injection of stigma constituent, crocin at concentrations of 0.1 and 0.2 g/kg significantly attenuated paw thickness and infiltration of neutrophils in paw tissues (Tamaddonfard et

al., 2012). The anti-inflammatory effect of crocin was also observed in neuroinflammatory diseases like multiple sclerosis (Deslauriers et al., 2011). Kumar et al. (2012) examined various petal extracts of *C. sativus* Cashmerianus to assess anti-inflammatory effect by carrageenan-induced paw edema method. Among all the extracts, methanol extract (400 mg/ml) exhibited 63.16% inhibition of paw volume followed by aqueous (57.89%) and chloroform (50%) extracts. Phytochemical profile of *C. sativus* stigma and petal suggests that both the anti-inflammatory and analgesic properties of the plant might be related to its carotenoid and flavonoid constituents.

2.8.6 Antidepressant effect

Depression is a mental illness that was often considered a sign of tiredness or weakness rather than a health problem that might need urgent medical treatment (Razak et al., 2017). Synthetic antidepressants have shown some adverse reactions in the patients i.e., sexual dysfunction, constipation, dry mouth (Khawam et al., 2006) and thus herbal medicines including saffron are a better alternative for being more safe, tolerable and more acceptable to people (Lopresti & Drummond, 2014). Hosseinzadeh et al. (2007) evaluated the antidepressant activity of a *Crocus sativus* petal constituent, kaempferol and highlighted that it significantly attenuated immobility behaviors in rats and mice and showed an almost similar response to fluoxetine. Similarly, in an 8-week double-blind randomized clinical study, dried saffron petal (15 mg bid) had similar antidepressant effects as fluoxetine (15 mg bid) in treating patients with mild-to-moderate depression and no significant differences in observed side effects (Basti et al., 2007). In an investigation comparing the efficacy of saffron stigma and corms with fluoxetine against depression, petroleum ether and dichloromethane fractions of saffron stigma and corms significantly reduced the immobility time in the tail suspension and forced swimming test at all doses (150, 300, and 600 mg/kg) without altering the locomotor behavior of mice during the open-field test. The authors highlighted that antidepressant effect of stigma

extracts could be due to crocin analogs, particularly crocin 1. However, HPLC analysis of corm extract revealed the absence of safranal, crocin, crocetin, or kaempferol compounds assuming the presence of other bioactive compounds in saffron corms showing a potent antidepressant effect, which need to be further explored (Wang et al., 2010).

2.8.7 Anticoagulant effect

Anticoagulant drugs such as heparins, vitamin K-antagonists, and their derivatives have been used in the clinical setting for over six decades. However, the life-threatening adverse reactions of these drugs have also been well studied (Bounameaux, 2009; Stone et al., 2007). Medicinal plants serve as the alternative sources for the development of new anticoagulant agents due to their potent pharmacological activities. Several scientific evidence demonstrate that the consumption of saffron dietary anticoagulants can reduce the possible risks associated with cardiac problems (Ayatollahi et al., 2014; Shiping et al., 1999; Thushara et al., 2013). Crocin delayed blood clotting time and mitigated respiratory distress as a result of pulmonary thrombosis in mice, inhibited thrombosis in rats, and suppressed platelet aggregation in rabbits (Shiping et al., 1999). Crocetin significantly reduced collagen- and ADP-induced platelet aggregation but failed to reduce arachidonic acid-induced platelet aggregation (Yang & Qian, 2007). Besides that, crocetin significantly reduced dense granule secretion, while neither platelets adhesion to collagen nor cyclic AMP level was affected by crocetin (Yang et al., 2008). Saffron stigma tablets (200 and 400 mg/day) assessed for short-term safety and tolerability in a limited number of volunteers showed that only 200 mg of saffron tablets reduced International Normalize Ratio, platelets, and coagulation time (Modaghegh et al., 2008). Later, in a double-blind, placebo-controlled clinical study with a large sample size, saffron tablets (200 and 400 mg/day) administration failed to show any major effect on coagulant and anticoagulant system after one month. The authors suggested that the case reports of hemorrhagic

complications might be due to high saffron dose, high period of consumption, or idiosyncrasy activities (Ayatollahi et al., 2014).

2.8.8 Anticancer effect

Cancer is a serious public health disorder globally, claiming more than 8 million lives annually (Torre et al., 2015). Moreover, approximately 1 million new cases of cancer and about 0.5 million cancer deaths reported in the US alone in 2015 (Siegel et al., 2015). The prevalence of undesired adverse effects linked to the use of conventional-therapeutic techniques (surgery, chemotherapy, radiotherapy, and immunotherapy) and even recently designated drug therapies mediated against specific cancer targets, have compelled the researchers to explore alternative treatment approaches (Wang et al., 2012). Previous reports suggest that several dietary-compounds, such as saffron, ginger, and garlic, exhibit promising anticancer and chemopreventive effects without causing the side effects accompanied with the use of synthetic drugs (Bayan et al., 2014; Milajerdi et al., 2016; Shukla & Singh, 2007). Various anticancer properties of saffron and its key ingredients are summarized in Table 2.3.

2.8.8.1 Breast cancer

The natural spice saffron has gained profound interest on account of its biocompatible nature and substantial anti-cancer properties, based upon several *in vitro* and *in vivo* reports. *In vitro* experiments have shown that amongst the various phytochemicals extracted from the spice, crocin holds the greatest anticancer potential. For example, studies carried out as early as 1996 on the HeLa cell line showed that crocin exhibited the greatest cancer inhibitory potential in contrast to the other phytochemical compounds, such as crocetin, picrocrocin, and safranal. The said results were concluded by observing the differences in cellular size, cytoplasmic volume and other morphological changes before and after treatment with the compounds (Escribano et al., 1996). Chryssanthi et al.

(2007) further investigated the anti-proliferative property of crocin on two breast cancer cells MDA-MB-231 and MCF-7 and found that crocin extracted from any species of *Crocus* is the main constituent responsible for anti-proliferation regardless of the degree of glycosylation, which was later supported by Mousavi et al. (2009).

2.8.8.2 Prostate cancer

D'Alessandro et al. (2013) evaluated the inhibitory potential of crocin and saffron extract on 5 malignant and 2 nonmalignant cells. The results indicated that both test compounds decreased cells proliferation of all malignant cells with no observable cytotoxic effects in non-malignant cells. Flow cytometry analysis indicated that most of the cells were arrested at the G_0/G_1 phase with a notable existence of apoptotic cells. The expression of Bax, a pro-apoptotic protein, was upregulated, whereas Bcl-2, an anti-apoptotic protein, was strikingly downregulated as per the analysis of Western blot. Moreover, the study of the enzymatic activity of caspase showed a caspase-dependent pathway with high levels of caspase-9, suggesting the activation of mitochondrial associated apoptosis (intrinsic mitochondria pathway). Similarly, preclinical research reported by Samarghandian & Shabestari (2013) revealed significant concentration-dependent cytotoxic effects of safranal against human prostatic carcinoma cell (PC-3) in contrast to the non-malignant cells.

2.8.8.3 Pancreatic cancer

Pancreatic cancer has the fourth highest rate of mortality among cancer patients having an average survival of 6 months and a dismal 5-year survival frequency between 3–5% (Iovanna et al., 2012). Regardless of new advancement in the current therapeutic methods, the mean 5-year survival frequency still remains less than 5% (Bhandari, 2015). Therefore, new therapeutic alternatives are intensely required for people suffering from pancreatic adenocarcinoma. During the last few years, a series of experiments have been

carried out both *in vitro* and *in vivo* to evaluate the influence of saffron constituents on the growth and proliferation of pancreatic cancers. Dhar et al. (2009) demonstrated the anti-tumorigenic activity of crocetin against pancreatic cancer using human pancreatic cancer cells such as Capan-1, ASPC-1, MIA-PaCa-2, BxPC3, and a xenograft athymic mouse model. The results revealed that crocetin suppressed the proliferation of pancreatic cancer cells and significantly reduced cell distribution within S-phase confirming impairment in DNA replication. Crocetin significantly altered cell cycle regulatory proteins such as Cyclin-B1, Cdc-25C, Cdc-2 and epidermal growth factor receptor (EGFR). During *in vivo* study, MIA-PaCa-2 cell lines were directly injected into xenograft athymic mice followed by oral treatment of crocetin, after palpable tumor development. The findings indicated a significant rise in proliferating cell nuclear antigen-positive cells in control samples as compared to crocetin-treated samples. Additionally, there was a significant decrease in EGFR expression and phosphorylation in mice treated with crocetin than in untreated samples (Dhar et al., 2009). Bakshi et al. (2010) further examined the anti-cancer properties of crocin on human pancreatic cancer cells Bx-PC-3 and found significant results. These findings show that crocetin has an effective antitumorigenic activity both *in vitro* and *in vivo* against pancreatic cancers.

2.8.8.4 Colorectal cancer

Colorectal cancer (CRC) accounts for 9.4% of all incident cancers globally, with approximately 1 million new cases are diagnosed yearly (Cantero-Muñoz et al., 2011). Due to the increase in knowledge of molecular biology of the disease, new therapeutic strategies have been developed in the last few decades. Aung et al. (2007) examined the antiproliferative assay of crocin and saffron extract on CRC cells such as HT-29, HCT-116, and SW-480 and suggested that they significantly suppressed the proliferation of cancer cell lines without any harm to normal cells. A similar experiment by Bajbouj et al. (2012) determined that saffron extract was effective in apoptotic induction in colorectal

cancer cells. They treated HCT116 human colon cancer cells (HCT p53−/− and HCT wildtype) with a saffron extract that resulted in DNA-damage and apoptotic cell death in both cancer cells. Hence, saffron should be examined further in detail as a viable drug to prevent the development of colorectal cancer.

2.8.8.5 Gastric cancer

Recent studies have unveiled the influence of saffron and its chemical components to combat the risks associated with gastric cancer. A study undertaken by (Bathaie et al., 2013) indicated that saffron aqueous extract has beneficial effects on 1-methly-3-nitro-1-nitrosoguanidine (MNNG)-induced gastric cancer in rats. Pathologic data indicated that saffron extract administration inhibited cancer progression in the gastric tissue in such a way that 20% of cancer-bearing rats administered with a higher concentration of saffron extract were found to be totally normal after clinical trials. In addition, the apoptosis/proliferation rate enhanced with the treatment of saffron extract to cancerous rats as indicated by flow cytometry analysis/propidium iodide staining. Thus the pharmacologists recommend saffron and its extract as a potential chemotherapeutic agent against gastric cancer (Bathaie et al., 2013; Hoshyar et al., 2013).

2.8.8.6 Lung cancer

The ethanolic and aqueous extract of saffron is also known to play a vital role as a tumoricidal agent in lung cancer, the second most common cancer in men after liver cancer. In order to determine the cytotoxic and antiproliferative potential of saffron in carcinomic human alveolar basal epithelial cells (A549), Samarghandian et al. (2010) proved that administration of saffron ethanolic extract significantly suppressed the growth of A549 cells in a dose and time-dependent manner compared with non-malignant (L929) cells. A similar study undertaken by Samarghandian et al. (2013) reported that the proliferation of A549 malignant cell lines decreased significantly following treatment

with aqueous extract of saffron in a concentration- and time-dependent manner. Furthermore, the frequency of apoptotic cells was also improved. This study strongly suggests that both ethanolic and aqueous extract of saffron could be used as a potential chemopreventive drug for lung cancer (Samarghandian et al., 2013; Samarghandian et al., 2010; Samarghandian et al., 2011).

2.8.8.7 Liver cancer

The chemoprevention of hepatocellular carcinoma (HCC), the third most frequent cause of cancer-associated mortality, is a promising approach against its development and metastasis. Natural herbs and plants like saffron have got immense attention for its role as an antiproliferative and proapoptotic agent in different hepatocellular cancer cell line. In the study undertaken by Amin et al. (2011), saffron administration to diethylnitrosamine (DEN)-treated rats significantly decreased the number and frequency of hepatic dyschromatic nodules and development of foci of altered hepatocyte (FAH). Additionally, treatment with saffron resulted in counteracted DEN-induced oxidative stress in an animal model as shown by reestablishment of antioxidant enzymes such as catalase (CAT), superoxide dismutase (SOD) and glutathione-S-transferase (GST) levels and lowering of malondialdehyde (MDA), myeloperoxidase (MPO) activity, and protein carbonyl production in the liver. In another study, Tavakkol-Afshari et al. (2008) investigated the cytotoxic and apoptogenic activity of 96% saffron ethanolic extract in malignant (HeLa, HepG2) and non-malignant (L929) cells. They found that saffron extract reduced cell viability in both malignant cell lines in a dose- and time-dependent manner than in L929 cells. Recently, Amin et al. (2016) studied the anti-tumorigenic activity of crocin in HCC using human liver cancer cell line (HepG2). Their findings showed anti-proliferative and pro-apoptotic characteristics of crocin when administrated in HCC-induced rats. Based on these results, saffron in combinations with other

commonly used chemotherapeutic drugs could be used against liver cancer (Amin et al., 2016; Amin et al., 2011; Tavakkol-Afshari et al., 2008).

2.8.8.8 Skin cancer

Over the past several decades, pharmacists are interested in understanding the cellular and molecular mechanism of skin cancer and discover the substances to be used in its chemoprevention. Fortunately, these days the chemoprevention of cancer is known to be the most hopeful and innovative strategy to suppress or inverse the tumorigenesis process using different natural products or plants (Bhandari, 2015). *In vitro* findings have reported that crocin can slow down the development of mouse skin papillomas as compared to other carotenoid pigments of saffron tested such as crocetin di-glucose ester and crocetin gentiobiose glucose ester (Konoshima et al., 1998). The chemopreventive potential of aqueous saffron extract on two-stage skin carcinogenesis in albino mice started by 7,12 dimethylbenz[a]anthracene (DMBA) followed by croton oil was assessed. Standard histological analysis of skin revealed significant suppression in papilloma formation where saffron was administered before and after the induction of skin papillogenesis. The suppression of skin papillogenesis could be attributed to the modulatory activities of saffron on Phase II detoxifying enzymes namely glutathione peroxidase (GPx), SOD, CAT and GST (Das et al., 2004; Das et al., 2010). In another experiment, topical administration of saffron extract (100 mg/kg body weight) suppressed two-stage initiation/promotion (DMBA/croton oil) skin papillogenesis and the similar concentration of saffron extract inhibited the action of 20-methylcholanthrene (MCA)-induced soft-tissue sarcomas in mice upon oral administration (Salomi et al., 1991). More research is required to further examine the mode of action of saffron to make it a potential therapeutic agent against skin cancer.

2.8.8.9 Leukemia

Numerous experiments have illustrated that saffron and its characteristic components inhibit the proliferation and cancerogenicity of leukemic cells. According to Rezaee et al. (2013) significant reduction in growth and viability of human T-cell leukemia cells MOLT-4 and an increase in DNA fragmentation was recorded at higher doses of crocin treatment. It was demonstrated that mild cytotoxic activities of crocin on MOLT-4 might be mediated by DNA fragmentation. *In vitro* curative experiments have proved that crocin suppressed the proliferation of Human leukemia HL-60 cell line and induced apoptosis and cell cycle arrest at G0/G1 phase, in a dose and time-dependent manner (Sun et al., 2013). To assess the *in vivo* effect of crocin on human leukemia, nude mice xenograft model was used, and the results demonstrated that crocin suppressed the tumor weight and size of HL-60 cells in mice. Moreover, it inhibited the expression of Bcl-2 and improved Bax expression in xenografts (Sun et al., 2013). Besides that, Makhloufa et al. (2016) tested the anti-proliferative effect of Lebanese saffron on human acute lymphoblastic T-cell leukemia (Jurkat cells) and found that saffron extract and a mixture of its components (safranal and crocin) reduced the proliferation of Jurkat cells dose-dependently. However, the IC_{50} value of the whole saffron extract was higher than that of its mixture. These findings suggest that saffron and its characteristic components could be used as a viable option against leukemia in clinical trials (Geromichalos et al., 2014).

Table 2.3: A summary of anticancer activities of *C. sativus* and its key components

Type of cancer	Saffron constituent	Cell lines/ animal models	Result	Reference
Breast cancer	Crocin and crocin with hyperthermia	MDA-MB-468 cells	Combination of crocin with hyperthermia increased mRNA ratio of Bax/Bcl-2 more than crocin alone.	Mostafavinia et al. (2016)
	Crocetin, safranal, and trans-crocin-4,	MCF-7 and MDA-MB-231 cells	All three constituents suppressed the proliferation of both cell lines but the antiproliferative activity of saffron was ascribed to crocins regardless of the degree of glycosylation.	Chryssanthi et al. (2007)
	Crocin	MCF-7 cells	IC_{50} of crocin was 60 µg/mL and 12.5 µg/mL at 24 and 48 h.	Lu et al. (2015)
	Combination of crocin with paclitaxel or gamma radiation	MCF-7 cells	1.5-6 mg/mL crocin inhibited the proliferation with a dose-time dependent manner. The collective effect of crocin with paclitaxel or radiation displayed a synergistic effect on MCF-7 cells.	Vali et al. (2015)
Cervical cancer	Saffron extract, crocin, and picrocrocin	Malignant TC-1 and non-malignant COS-7 cells	IC_{50} of crocin, picrocrocin and saffron extract on TC-1 cell line as 1.5 mM, 3 mM and 4 mg/mL, respectively confirmed crocin as the main growth inhibiting effect in saffron extract	Alizadeh & Bolhassani (2015)
	Crocetin	HeLa cells, A549 cells, ovarian cancer SKOV3 cells, and vincristine-resistant breast cancer MCF-7/VCR cells	60-240 µmol/L crocetin suppressed cells proliferation dose-dependently by inducing G1 arrest through p53-dependent and –independent pathway assisted with p21WAF1/Cip1	Zhong et al. (2011)
	Saffron ethanolic extract, crocetin, crocin, picrocrocin and safranal	HeLa cells	IC_{50} values of saffron extract, safranal, picrocrocin and crocin against HeLa cells were 2.3 mg/mL, 0.8 mM, 3 mM, and 3 mM, respectively. Crocetin failed to show cytotoxic activity.	Escribano et al. (1996)
Ovarian cancer	Crocin	Ovarian cancer HO-8910 cells	Inhibition of cell proliferation and apoptotic induction by increasing the expression of p53, Fas/APO-1, and Caspase-3.	Xia (2015)
Prostate cancer	Saffron extract and crocin	Human prostate cancer malignant cells LAPC-4,	Both saffron extract and crocin showed a reduction of proliferation in all malignant cells with no cytotoxic effect on	D'Alessandro et al. (2013)

Table 2.3, Continued.

		CWR22, LnCaP, 22rv1, C4–2B, DU145 and PC3 and nonmalignant cells BPH-1 and EPN	nonmalignant cells. The expression of Bax was upregulated, whereas Bcl-2 was downregulated.	
	Safranal	PC-3 cells and human fetal lung fibroblast MRC-5 cells	Inhibition of proliferation of PC-3 cell lines in a concentration- and time-dependent manner. No significant effect of low concentration of safranal after 24 h however, IC_{50} dose of safranal for PC-3 cells was 13 μg/mL and 6.4 μg/mL at 48 and 72 h, respectively.	Samarghandian & Shabestari (2013)
	Saffron extract, crocetin, and crocin	PC3 and 22rv1 cells in male nude mice	Strongest antitumor activity in PC3 and 22rvl xenografts showed by croctein compared to saffron extract and crocin. Conversely, saffron extract and crocin induced stronger epithelial differentiation.	Festuccia et al. (2014)
Pancreatic cancer	Crocetin	Human pancreatic cancer cell MIA-PaCa-2, BxPC-3, Capan-1 and ASPC-1 Xenograft athymic female mouse model	Apoptosis was significantly stimulated by crocetin in both *in vitro* pancreatic cancer cell lines and *in vivo* athymic mice tumor, as directed by Bax/Bcl-2 ratio.	Dhar et al. (2009)
	Crocin	BxPC-3	Apoptotic induction and cell cycle arrest of BxPC-3 cells at G1-phase while reduction of cell viability in a dose and time-dependent manner.	Bakshi et al. (2010)
Colorectal cancer	Saffron extract	Human CRC cells HCT116 (HCT wildtype and HCT p53−/−)	Saffron extract induced DNA-damage and apoptosis in both cancer cells. Also, apoptotic induction in HCT116 p53 −/− cells was delayed by autophagy.	Bajbouj et al. (2012)
	Saffron extract and crocin	Human colorectal cancer cells HCT-116, HT-29, SW-480, human non-small cell lung cancer cells, and young adult mouse colon cells.	Saffron extract and crocin exhibited the most potent anti-proliferative activity against HCT-116 cells than the other two cells.	Aung et al. (2007)

Table 2.3, Continued.

	Crocin	Male ICR mice	Inhibition of azoxymethane/dextran sodium sulfate (AOM/DSS)-induced colitis and DSS-induced colitis in mice by inhibiting cytokines expression	Kawabata et al. (2012)
Gastric cancer	Crocetin	Gastric adenocarcinoma AGS cell lines and human normal fibroblast cell lines HFSF-PI3, Male Wistar albino rats	Inhibition of Bcl-2 expression and up-regulation of Bax expression in cancer cell lines.	Bathaie et al. (2013)
	Saffron aqueous extract	Male Wistar albino rats	Inhibition of the proliferation of cancer cells dose-dependently.	Bathaie et al. (2013)
	Crocin	AGS and HFSF-PI3 cell line	2.2-3.5mg/mL crocin improved the cells percentage in the sub-G1 phase up to 60% after 24 h.	Hoshyar et al. (2013)
	Crocetin	Human gastric cancer BGC-823 cell	Reduction of Mitochondrial membrane potential of crocetin-treated BGC-823 cells in a concentration and time-dependent manner.	He et al. (2014)
Lung cancer	Crocin	A549 and SPC-A1	Suppression of proliferation and apoptotic induction in cancer cell lines in a dose-dependent manner	Chen et al. (2015)
	Saffron aqueous extract	A549 and human fetal lung fibroblast MRC-5 cells	IC_{50} of the saffron extract against A549 cells was 380 µg/mL and 170 µg/mL at 48 and 72 h, respectively.	Samarghandian et al. (2013)
	Crocetin	Male Swiss albino mice	Crocetin scavenged free radicals and increased the activity of drug metabolizing enzymes.	Magesh et al. (2006)
Liver cancer	Saffron ethanolic extract	Liver cancer cell HepG2, Rats	Inhibition of both nodular and foci of altered hepatocytes formation in livers of rats treated with diethylnitrosamine.	Amin et al. (2011)
	Crocin	Liver cancer cell line HepG2, Rats	Inhibition of inflammatory markers and reducing the viability of HepG2 cells by arresting cell cycle, apoptotic induction and downregulating inflammation.	Amin et al. (2016)
	Saffron ethanolic extract	Male Wistar rats	Enhancement of GST activity and reduction of MDA level in liver and serum nitric oxide (NO).	Samarghandian et al. (2016)

Table 2.3, Continued.

	Crocetin	Male Wistar rats	Protection of hepatic tissue against aflatoxin B_1 induced carcinogenicity	Wang et al. (1991)
Skin cancer	Crocin	Female ICR mice	Crocin delayed papilloma's formation in mice	Konoshima et al. (1998)
	Saffron extract	Male Swiss albino mice	Saffron extract suppressed onset and progression of induced skin carcinoma and delayed papilloma formation	Salomi et al. (1991)
	Crocetin	Mice fibroblast NIH/3T3 cells	120 and 60 µM crocetin inhibited the activity of TPA-induced protein kinase C by 66% and 50%, respectively	Wang et al. (1996)
	Aqueous saffron extract	Female Swiss albino mice	Saffron treatment before and after DMBA application reduced papilloma formation	Das et al. (2004) Das et al. (2010)
Leukemia	Crocin	Human T-cell leukemia MOLT-4 cells	Crocin at higher doses decreased cell viability with elevation in DNA fragmentation of MOLT-4 cells	Rezaee et al. (2013)
	Whole saffron extract, safranal, and crocin	Human T lymphocyte Jurkat cells	IC_{50} values of whole saffron extract and mixture of crocin and safranal against Jurkat cells were 71 µM and 39 µM, respectively	Makhloufa et al. (2016)
	Crocin, Crocetin, and dimethylcrocetin	Promyelocytic leukemia HL-60 cells	Crocin, Crocetin, and dimethylcrocetin inhibited the growth of HL-60 cells with IC_{50} values of 0.8 µM, 2 µM and 2 µM, respectively.	Tarantilis et al. (1994)
	Crocin	Human leukemia HL-60 cells, Male nude BALB/c mice	6.25 and 25mg/kg crocin showed strong inhibitory effect on HL-60 along with Bcl-2 expression and improved Bax expression in xenografts	Sun et al. (2013)

2.8.9 Effect on Cardiovascular diseases

Cardiovascular diseases (CVDs) are the major threats to global health, claimed an estimated 17.5 million lives in 2012 (WHO, 2013). Globally, the number of CVDs deaths elevated from 12.3 million (25.8%) in 1990 to 17.3 million (31.5%) in 2013 (Naghavi et al., 2014). The high mortality rate of CVDs highlights the need of an effective approach for its treatment. Recent experiments have shown several therapeutic properties of saffron in the effective treatment of many cardiovascular-related disorders, including atherosclerosis, hyperlipidemia, hypertension, diabetes mellitus and myocardial ischemia, as summarized in Table 2.4.

2.8.9.1 Atherosclerosis

Liu & Qian (2005) evaluated the preventive effect of crocin on cholestane-3β, 5α, 6β-triol-induced apoptosis along with the gene expression patterns of cultured endothelial cells and found that crocin inhibited apoptosis of endothelial cells by improving the gene expression of Bcl-2 and decreasing Bax and Caspase-3 gene expression. Further studies confirmed that the antiapoptotic property of crocin plays a key role in the prevention and regression of atherosclerosis (Xu et al., 2007). Crocetin and crocin also caused antiatherosclerotic effects in quail by reducing the level of Ox-LDL that induces gene expression in endothelial cells resulting in progression of atherosclerosis (He et al., 2005; He et al., 2007).

2.8.9.2 Hyperlipidemia

High level of lipids is considered as a primary factor involved in the development of CVD. Currently, pharmacologists are focusing on the novel drugs with the capability to reduce and regulate cholesterol and TG content of serum. Sheng et al. (2006a) examined the hypolipidemic property of crocin extracted from *Gardenia jasminoides* Ellis in rats and indicated that the compound significantly lessened serum TG, total, LDL and VLDL

cholesterol level in experimental models. The authors added that the hypolipidemic activity of crocin attributed to its suppression of pancreatic lipase activity. Similar findings were obtained by Lee et al. (2005) where both crocetin and crocin isolated from *G. jasminoides* improved hyperlipidemia. In further studies, saffron was reported to be more effective in responding to hyperlipidemia manifestation than crocin suggesting the involvement of another active saffron components other than crocin in synergistic antihyperlipidemic and antioxidant activity of saffron (Asdaq & Inamdar, 2010).

2.8.9.3 Hypertension

Hypertension has long been known as a serious risk factor for cardiovascular diseases and mortality. Over the past two decades, studies have shown that most of the people living in developing countries experience higher incidences of hypertension (Bromfield & Muntner, 2013). Therefore, the detection of hypertension and its control is critically important to overcome the possible risk of heart attacks and strokes. Globally, many traditional medicinal plants, including saffron, have been reported to be anti-hypertensive (Imenshahidi et al., 2010; Imenshahidi et al., 2013; Llorens et al., 2015; Nasiri et al., 2015; Rawat et al., 2016). In an investigation comparing the hypotensive activity of aqueous extract of saffron and the compounds, crocin and safranal against normotensive and desoxycorticosterone acetate-induced hypertensive rats revealed that both the saffron extract and compounds reduced the MABP of rats dose-dependently The authors further suggested safranal as the principal constituent to the anti-hypertensive effect of saffron extract (Imenshahidi et al., 2010). Similarly, treatment of aqueous extract of saffron (0.01, 0.02 and 0.04 g/kg/day) significantly decreased MSBP in DOCA-salt-induced hypertensive rats dose-dependently. However, the hypotensive activity of saffron extract did not persist, as SBP started to increase after stopping the saffron administration (Imenshahidi et al., 2013). Nasiri et al. (2015) highlighted the nutritional effect of hydroalcoholic extract of saffron on blood pressure and histology of aorta and

demonstrated that saffron showed no activity against normotensive rats but prevented BP elevation, decreased the aortic cross-sectional area, tunica media thickness, and elastic lamellae number in hypertensive rats. In another study, crocetin, and to some degree crocin, reduced the maximum contractile effect to phenylephrine in intact rings, and crocin improved contractility in de-endothelizated vessels. Additionally, crocetin activity on contractility was not affected by indomethacin in the intact vessels but was eliminated by L-NAME (an inhibitor of nitric oxide synthase) (Llorens et al., 2015).

2.8.9.4 Diabetes mellitus

Diabetes mellitus, a chronic disorder characterized by high blood pressure with alteration in metabolism of carbohydrates, fats, and proteins, is one of the major factors affecting the renal, retinal, and nervous system. It is closely associated with hyperlipidemia and hypertension that results in cardiovascular morbidity and mortality. Several reports showed the possible inhibitory effect of saffron against complication of diabetes such as oxidative stress, hyperglycemia, and hyperlipidemia (Altinoz et al., 2015; Samarghandian et al., 2014; Samarghandian et al., 2013; Shirali et al., 2013). Administration of safranal after streptozotocin (STZ) treatment significantly decreased blood glucose level, nitric oxide, serum malondialdehyde (MDA), total lipids, triglycerides and cholesterol level with elevation in the level of glutathione (GSH), catalase and superoxide dismutase (SD) activity in the safranal-treated diabetic rats (Samarghandian, Borji, Delkhosh, et al., 2013). In another experiment by the same team revealed that saffron extract significantly improved GSH content, catalase, and SD activity but significantly reduced serum TNF-α, cognitive deficit, and induced activity of nitric oxide synthase in hippocampus tissue of diabetic rats. Resultantly, saffron reduced the possible risk of hyperglycemia, hyperlipidemia and oxidative stress in diabetic rats (Samarghandian et al., 2014). In another experiment, crocin administration after STZ treatment enhanced histopathological changes in heart tissue and reduced MDA levels

with elevation in GSH levels of both serum and heart tissue (Altinoz et al., 2015). Overall, these findings show that saffron and its active constituents might inhibit diabetes induced CVD by modulating oxidative stress and dyslipidemia.

2.8.9.5 Myocardial ischemia

Myocardial ischemia, caused by a critical coronary artery obstruction, is among the most common coronary heart diseases resulting in higher morbidity and mortality worldwide, especially in the Western world. Several natural products including saffron have been tested for their activity against cardiac ischemia and it is suggested that orally administered crocetin is a promising anti-myocardial ischemia drug in clinical practice. An *in vivo* rat model of myocardial injury revealed that crocetin reduced the secretion of lactate dehydrogenase (LDH), creatine kinase (CK) and MDA levels, whereas enhanced the level of SOD and cardiac myocytes activity in rats. However, serum containing crocin showed no remarkable improvement in myocardial injury. The authors remarked that crocin is barely absorbed through the gastrointestinal tract following oral administration and its quantity in serum is very low (Zhang et al., 2009). Likewise, saffron extract and safranal showed cardioprotective activity in isopropanol (ISO)-induced myocardial infraction by modulation of oxidative stress. Pretreatment of saffron extract and safranal reduced the serum CK-MB, LDH and myocardial lipid peroxidation in comparison with ISO-induced rats (Mehdizadeh et al., 2013). Besides that, Huang et al. (2016) demonstrated the potential of crocetin ester in the reduction of serum contents of pro-inflammatory cytokines along with CK, MDA, LDH and SOD activities. In addition, crocetin ester ameliorated the histopathological alteration. The authors assumed that ISO-induced acute myocardial ischemia could be possibly treated by crocetin ester via the inhibition of the Rho/ROCK/NF-κB pathway.

Table 2.4: Various pharmacological activities of *C. sativus* and its main constituents against cardiovascular diseases

CVDs	Saffron constituent	Cell lines/ animal models	Result	Reference
Atherosclerosis	Crocin	Human endothelial cell	Elevation of Bcl-2 expression and reduction of Bax gene expression and Caspase-3 activity.	Liu & Qian (2005)
	Crocetin	Male white rabbits	8-week treatment with crocetin suppressed the expression of vascular cell adhesion molecule-1 (VCAM-1) and meliorated atherosclerosis in rabbits	Zheng et al. (2005)
	Crocin (from *G. jasminoides*)	Bovine aortic smooth muscle cell, bovine aortic endothelial cell and male quails	9-week treatment with crocin lessened Ox-LDL level that induces gene expression in endothelial cells thus suppressed atherosclerosis production in quails	He et al. (2005)
	Crocetin (from *G. jasminoides*)	Male quails	9-week treatment with crocetin lessened serum triglycerides (TG), total and LDL cholesterol levels, and suppressed aortic plaque development along with reducing malonaldehyde, and thwarted decreased level of serum nitric oxide	He et al. (2007)
	Saffron crude extract and crocin	Human coronary artery endothelial cells (HCAEC)	Treatment of HCAECs with saffron and crocin reduced protein expression of sICAM-1 and sVCAM and gene expression	Alicezah et al. (2014)
Hyperlipidemia	Crocin from *G. jasminoides*	Male Sprague–Dawley rats	10-day treatment with crocin decreased serum TG, total and LDL as well as VLDL cholesterol level	Sheng et al. (2006a)
	Crocetin and crocin from *G. jasminoides*	Male ICR mice	Both crocetin and crocin suppressed serum TG, total and LDL cholesterol level	Lee et al. (2005)
	Saffron and crocin	Albino Wistar rats	High saffron dose showed more effectiveness in counteracting the manifestation of hyperlipidemia than high crocin dose	Asdaq & Inamdar (2010)
	Saffron aqueous extract	Male Albino Wistar rats	Saffron extract dose dependently decreased serum TG, LDL, total lipids, and cholesterol level but increased serum HDL level	Samarghandian et al. (2014)

Table 2.4, Continued.

Hyperglycemia	Crocin	Neonatal male Wistar rats	9-week treatment with crocin lessened serum glucose, total and LDL cholesterol, TG, advanced glycation end products levels and improved HDL in diabetic rats	Shirali et al. (2013)
	Safranal	Albino Wistar rats	Safranal dose-dependently decreased blood glucose, MDA, TG, NO, total lipids and cholesterol and increased GSH level as well as CAT and SOD activity	Samarghandian et al. (2013)
	Saffron aqueous extract	Male Albino Wistar rats	Elevation of body weight and serum TNF-α but reduction in blood glucose level and serum advanced glycation end products levels along with glycosylated serum proteins.	Samarghandian et al. (2014)
	Saffron methanolic extract, crocin and safranal	Male adult Wistar rats	Reduction in blood glucose and HbA1c level but elevation in the insulin content of blood with no significant effects on blood SGPT, SGOT and creatinine level	Kianbakht & Hajiaghaee (2011)
	Crocetin (from *G. jasminoides*)	Male Wistar rats	Crocetin thwarted dexamethasone-induced insulin resistant and enhanced insulin sensitivity in rats fed fructose by regularizing protein and mRNA expression of TNF-α, adiponectin and leptinin epididymal white adipose tissue.	Xi et al. (2005), Xi, Liang et al. (2007)
	Crocin	Female Wistar rats	Crocin ameliorated histopathological damages in heart tissue and decreased the level of MDA, TG, total and VLDL cholesterol levels, accompanied by an increase in GSH contents of both serum and heart tissue	Altinoz et al. (2015)
Anti-hypertension	Saffron extract, safranal, and crocin	Male Wistar rats	Saffron extract and constituents dose-dependently decreased mean arterial blood pressure in hypertensive and normotensive rats	Imenshahidi et al. (2010)
	Saffron aqueous extract	Adult male Wistar rats	Saffron extract dose-dependently decreased mean systolic blood pressure in desoxycorticosterone acetate (DOCA)-salt induced hypertensive rats	Imenshahidi et al. (2013)
	Crocetin and crocin	Male spontaneously hypertensive rats (SHR)	Crocetin reduced the maximum contractile effect to phenylephrine in intact rings, and crocin enhanced contractility in de-endothelizated vessels	Llorens et al. (2015)

Table 2.4, Continued.

	Saffron hydroalcoholic extract	Male Wistar rats	Saffron extract prevented BP elevation, decreased aortic cross-sectional area, tunica media thickness and number of elastic lamellae in hypertensive rats	Nasiri et al. (2015)
Myocardial ischemia	Crocetin and crocin	Male Sprague-Dawley rats	Crocetin protected the injured myocardial cell both *in vitro* and *in vivo* in comparison with crocin	Zhang et al. (2009)
	Saffron aqueous extract and safranal	Male Albino Wistar rats	Saffron extract (20-160 mg/kg/day IP) and safranal (0.025-0.075 ml/kg/day IP) reduced serum CK-MB and LDH activities along with myocardial lipid peroxidation dose-dependently	Mehdizadeh et al. (2013)
	Crocetin ester	Sprague–Dawley rats	Crocetin ester showed cardioprotection against isoproterenol (ISO)-induced acute myocardial ischemia by Rho/ROCK/NF-κB signaling pathway	Huang et al. (2016)
	Saffron extract	Rat embryonic ventricular cardiomyocyte cell line H9c2	Saffron extract activated the levels of phosphorylated AKT, 70S6 kinase and ERK1/2, recovered contractile proteins expression, inhibited alteration in mitochondrial morphology and reduced caspase-3 activity in H9c2 cardiomyocytes	Chahine et al. (2016)

MATERIAL AND METHODS

3.1 Micropropagation

3.1.1 Working Precautions

Sterilized conditions were maintained during seeds germination and tissue culturing in order to minimize the chances of contamination, cross contamination and for personal safety. For this purpose, following measures were taken.

- Lab coat or overall was used while in the laboratory.
- All materials used for culturing microorganisms were sterilized by autoclaving at 121°C for 20 minutes.
- Disinfectants such as 70% ethanol or 10% sodium hypochlorite (bleach solution) were used to swab benches and working areas.
- For surface sterilization, whole arrangement was exposed to ultraviolet radiations for 15-20 minutes.
- A disinfectant soap or hand sanitizer was used for washing hands before and after working with microorganisms.
- All waste materials were autoclaved or bleached and disinfected before their disposal.

3.1.2 Collection of plant material

Healthy and disease free *Crocus sativus* L. corms weighing between 10-12 g and certified to be pure breeding were obtained from saffron corm industry, safranor, France. Corms were stored in dark under normal laboratory conditions until further used.

3.1.3 Sterilization

3.1.3.1 Seeds surface sterilization

Prior to sterilization, tunic around the corm was removed by hand and thoroughly washed for 30 min under tap water to eliminate coating layer of microorganisms followed by rinsing in de-ionised water containing 3-4 drops of Tween-20 for 15 min. Thereafter, in aseptic conditions, the corms were treated with the sterilization procedures described below prior to washing thrice with autoclaved sterile distilled water.

(a) **Treatment 1**

15% H_20_2 treatment for 5 min followed by sterilization for 2-3 min with 70% ethyl alcohol and 5 min with 40% Clorox.

(b) **Treatment 2**

25% H_20_2 treatment for 5 min followed by sterilization for 2-3 min with 70% ethyl alcohol and 5 min with 40% Clorox.

(c) **Treatment 3**

70% ethyl alcohol treatment for 2-3 min followed by sterilization with 40% Clorox for 5 min.

(d) **Treatment 4**

70% ethyl alcohol treatment for 2-3 min followed by sterilization with 50% Clorox for 5 min.

(e) **Treatment 5**

70% ethyl alcohol treatment for 2-3 min followed by sterilization with 60% Clorox for 5 min.

(f) **Treatment 6**

70% ethyl alcohol treatment for 2-3 min followed by sterilization with 40% Clorox and 0.1% $HgCl_2$ for 5 min each.

(g) **Treatment 7**

70% ethyl alcohol treatment for 2-3 min followed by sterilization with 40% Clorox and 0.2% $HgCl_2$ for 5 min each.

(h) **Treatment 8**

70% ethyl alcohol treatment for 2-3 min followed by sterilization with 40% Clorox and 0.3% $HgCl_2$ for 5 min each.

3.1.3.2 Seeds sterilization medium

Murashige and Skoog medium (1962) without growth regulators was used as the basic media for seed sterilization experiment. To prepare one liter of MS basic media, a 1000 ml conical flask was used and filled up with 800 ml of distilled water. Then, 30 g/l sucrose, 4.4 g /l MS powder and 8 g/l technical agar were added into the conical flask. The media solution was stirred on a hot plate until all components were dissolved. After that, distilled water was added to reach the final volume of one liter. The pH of the media solution was adjusted to 5.8 using 1N hydrochloric acid (HCl) and 1N sodium hydroxide (NaOH). The media was autoclaved for 20 min at 121 ºC and 15 psi. After the media was autoclaved and cooled at 50 ºC, it was dispensed into 60 ml sterile universal containers in a LFH.

3.1.4 Direct regeneration of *C. sativus*

3.1.4.1 Bud sprouting

The sterilized corms were blot-dried with sterile paper and cultured onto MS (Murashige & Skoog, 1962) and Gamborg (Gamborg et al., 1976) medium with two concentrations of NAA (1, 2 mg/l) with Kn (1, 2, 4 and 6 mg/l) or BAP (1, 2, 4 and 6 mg/l) and IAA (1, 2 mg/l) with Kn (1, 2, 4 and 6 mg/l) or BAP (1, 2, 4 and 6 mg/l) in combinations were used for bud sprouting response. The pH was adjusted to 5.6-5.8 with HCl (1N) or NaOH (1N) before autoclaving for 20 min at 121 ºC and 15 psi. The cultures were incubated at temperature (24 ± 1 ºC) under photoperiod (16/8 h) illuminated with white fluorescent tube at irradiance of nearly 40 µmol m^{-2} s^{-1}.

3.1.4.2 Induction of shoot primordia

After 7 weeks, sprouted cultures were subsequently shifted to MS media supplemented with IAA (1, 2 mg/l) in combination with Kn (2, 4 and 6 mg/l) or BAP (2, 4 and 6 mg/l) and incubated at 24 ± 1 ºC and 16/8 h photoperiod for the induction of multiple shoot primordia.

3.1.4.3 Shoot multiplication

The clumps of shoot primordia formed were further incubated on MS media containing cytokinins (BAP and Kn) in different concentrations (2, 4, 6, 8 mg/l) with IAA (1, 2 mg/l) and kept in growth room under *in vitro* conditions as mentioned above to get multiple shoots. The cultures were replenished with same fresh medium to ensure the continuity of freshness every 4 weeks.

3.1.5 Cormlet production

The multiple shoots obtained were further transferred to MS medium containing IAA (1 mg/l) for cormlet production using varied concentration of filter sterilized GA_3 (1, 2 and 4

mg/l) and sucrose (3.0, 4.0 and 5.0%) under *in vitro* condition. Medium devoid of PGR and sucrose was used as a control. The weight of cormlets per explant was calculated by counting each forming corms from the total number of cormlets after 10 weeks of incubation. The number of cormlets produced per mother corm was also recorded.

3.1.6 Statistical Analysis

The ANOVA was achieved for all experiments and means of each experiment were statistically analyzed by Duncan's multiple range test (DMRT) at $p<0.05$ through SPSS statistical package (version 20). The results were expressed as mean ± SEM.

3.2 Agro-Environmental factors affecting saffron production

3.2.1 Site description

The current study was conducted in plant tissue culture room of Institute of Biological Sciences, University of Malaya, Kuala Lumpur Malaysia during 2016–2017 and 2017–2018 growing seasons.

3.2.2 Experimental design

The experiment was laid out in two years i.e., 2016–2017 and 2017–2018 in complete randomized design with three replications. The following factors and levels were tested: (i) corm weight grades (CW), i.e., small corms (10 g) and bigger corms (15 g); (ii) incubation temperature i.e. 18 °C and 24 °C and (iv) planting depth (PD), i.e. 8 cm and 16 cm, respectively.

3.2.3 Soil analysis

In terms of the soil characteristics, the soil texture of experimental black soil was a clay-loam structure containing 2.74% of total organic carbon and 0.04% of total nitrogen, in

addition to 7 ppm of phosphorus, 331.5 ppm of potassium, 64 ppm of calcium, 75.6 ppm of magnesium, and 25.3 ppm of sodium. The soil acidity was indicated at a pH of 7.5 at a depth of 0-20 cm.

3.2.4 Agronomic practices

The cultivation practices used were those commonly applied for saffron production (Seyyedi et al., 2018). Corms were planted with regular spacing in 7 cm distance within the rows and 10 cm distance between the rows in rectangular plastic trays measuring about 28 x 90 cm. A total of 8 corms were planted in each pot. The corms were incubated under a temperature-controlled room and 16 h photoperiod illuminated with white fluorescent tubes at an irradiance of nearly 40 μmol m^{-2} s^{-1}.

3.2.5 Water requirement

With regard to the irrigated production system, water was applied in August and September in order to improve the emergence of corms dormancy, and in March and April, to favor the production of daughter corms. However, in the first growing season, no water was applied between leaf withering and a month before the first buds of the replacement corms had grown.

3.2.6 Harvesting practices

Sampling started once flowering stage commenced. Flowers were collected and counted daily from late October to November, and then were transferred to laboratory and fresh weight of flower was recorded. Afterwards, stigmas were separated from the flowers by hand, weighed on an analytical scale and were dried at room temperature (25 °C) for 2-3 days, which is the traditional Iranian method widely used by saffron farmers in Iran, while the residual plant materials were dried in the oven (75 °C) for 2 days. The main reason of

choosing the traditional drying method for dehydration of stigma was to make saffron process similar to what farmers usually do. Total dry weight of stigma was recorded per plant. After the harvest period of stigma, the plants were left with green leaves for the multiplication of corms. Daughter corms produced were lifted at the end of the growing season in late May 2017 and related parameters such as weight and number were determined. All measured corms had a healthy appearance and of commercial value, with no wounded or unhealthy parts. During the experiment, observations for each parameter were monitored thrice per week and morphological abnormalities were also observed (if any). The same procedure was applied for the next year.

3.2.7 Statistical analysis

Data was recorded on different vegetative, floral, and corm attributes of saffron and subjected to statistical analysis using SPSS software. Two-way ANOVA was performed for each experiment and DMRT test was used to compare means at 0.05 level of probability. The results were expressed as mean ± SEM.

3.3 Induced gamma irradiation on floral and vegetative characteristics of saffron

3.3.1 Plant materials

Healthy disease-free *Crocus sativus* L. corms weighing between 10 to 12 g, certified to be pure breeding were used in this experiment.

3.3.2 Gamma irradiation

Corms were divided into 5 groups. One group was kept as the non-irradiated control sample and other groups were exposed to gamma irradiation at 10, 20, 30, and 40 Gy at a dosage rate of 1.65 Gy/min using a Gammacell 220 irradiator located at the Department of Physics, University of Malaya. Corms were stored at 18 ± 1 °C after irradiation.

3.3.3 Experimental design

The experiment was laid out in two separate experiments using complete randomized design with four replications and five treatments.

3.3.4 Growth conditions

The cultivation practices used were those commonly applied for saffron production (Seyyedi et al., 2018). The irradiated and non-irradiated control corms were planted 8 cm deep with regular spacing in 7 cm distance within the rows and 10 cm distance between the rows on 22 August 2018 in rectangular plastic trays measuring about 28 x 90 cm containing black soil. A total of 8 corms were planted in each row. Before corm planting, a basal organic fertilizer (mature manure) was scattered onto the soil surface as per requirement and then mixed into the soil. The corms were incubated under a temperature-controlled room at 18 ± 1 °C and 16 h photoperiod illuminated with white fluorescent tubes at the light intensity of 40 µmol m^{-2} s^{-1}. Selected physic-chemical characteristics of the experimental soil are given in Table 3.1.

Table 3.1: Physical and chemical analysis of the experimental soil

EC (dS m^{-1})	pH	Nutrient level							Soil texture
		OC (%)	N (%)	P (ppm)	K (ppm)	Mg (ppm)	Ca (ppm)	Na (ppm)	
1.08	7.58	2.74	0.04	7	331.5	75.6	64	25.3	Clay-loam

Soil texture class (0-20 cm)

3.3.5 Measurement of leaves, flowers, and corms

Data was recorded once the corm started sprouting in September. Data on the date of flowering was recorded by watching over the pots daily after 15 September and the days were counted to flowering. Flowers were then manually collected, counted, and weighed daily on an analytical scale from late October 2018 to late early December 2018 and the unit was given as harvest period or flower duration. Fresh stigmas were separated in the laboratory after flower-picking and weighed with an analytical scale. Afterward, stigmas were dried at 30 °C for 24 h in an oven (Koocheki & Seyyedi, 2015), while the residual plant materials were dried at 75 °C for 48 h. After the harvest period of stigma, the plants were left with green leaves for the multiplication of corms. Daughter corms produced were lifted at the end of the growing season in late May 2019 and related parameters such as weight and number were determined. All measured corms had a healthy appearance and of commercial value, with no wounded or unhealthy parts. During the experiment, observations for each parameter were monitored thrice per week and morphological abnormalities were also observed (if any).

3.3.6 Parameters

A total of 18 parameters were studied in each pot during the experiment. The descriptive data included corms survival rate, days to sprouting, sprouting rate, number of leaves, leaf length, plant height, days to flowering, number of flowers formed, number of flowers bloomed, flower duration, flower fresh weight, flower dry weight, stigma fresh weight, stigma dry weight, stigma length, floral shape variation, number of daughter corm and weight of daughter corm.

3.3.7 Statistical analysis

Data were recorded on different vegetative, floral, and corm attributes of saffron and subjected to statistical analysis using SPSS software. Two tests were performed i.e., ANOVA and DMRT at $p \leq 0.05$. The results were expressed as mean ± SEM.

3.4 Oral acute toxicity assay

3.4.1 Plant material

In vivo grown saffron plants components were used in the experiment. Different saffron parts were collected as per life span of the plant, i.e., petal and stigma were handpicked during October, leaves were collected in May, and smaller corms weighing less than 2 g were harvested in June. Individual parts were air-dried under shade for 7 weeks and ground to powder.

3.4.2 Sample extraction

Powder samples were macerated in ethanol/water (80%, v/v) at room temperature. The mixture was subsequently filtered with Whatman filter paper 1 (Sigma-Aldrich, St. Louis, MO, USA) and concentrated by rotary evaporator under reduced pressure at 45 °C. Semi-liquid extracts were further allowed to dry in the fume hood. The resulting crude extracts were stored in sealed tubes at −20 °C until used for the study.

3.4.3 Experimental animals

Adult albino mice (Swiss strain) of either sex with average age of 10 weeks, weighing between 25–30 g, were provided by Veterinary Research Institute (VRI) Peshawar, Pakistan. Mice were acclimatized in this environment for seven days before the experiments. All the experiments were carried out between 10:00 and 16:00. The study protocol (Bch 0275) for laboratory animals was in accordance with the guidelines of the Institutional Animal Ethics

Committee (QAU Islamabad). The mice were housed in aluminum cages (grade 304) under controlled natural 12/12 h light/dark cycle, temperature (25 ± 3 °C), and humidity (50–60%), and received tap water ad libitum and standard diet at the Animal House, Quaid-I-Azam University (QAU) Islamabad, Pakistan. All the precautionary measures were followed to reduce animal's fear and suffering.

3.4.4 Experimental design

The acute toxicity study was performed with respect to the Organization for Economic Cooperation and Development (OECD) guidelines 407 and 423 (OECD 407, 2008; OECD 423, 2001). A total of 50 mice were randomly divided into 5 experimental groups with 10 mice (5 males and 5 females) per group.

Group 1: Control (5% DMSO, 10 ml/kg)

Group 2: Corm ethanolic extract (CEE) - 2000 mg/kg

Group 3: Leaf ethanolic extract (LEE) - 2000 mg/kg

Group 4: Petal ethanolic extract (PEE) - 2000 mg/kg

Group 5: Stigma ethanolic extract (SEE) - 2000 mg/kg

A 2000 mg/kg dose of saffron ethanolic extracts was chosen for the limit test in accordance with OECD guidelines 407 and 423 for the testing of chemicals. Each of the ethanolic extracts of saffron was administered in mice as a single dose (2000 mg/kg) by oral gavage through a stainless-steel feeding needle at the start of the experiment, and the rats were monitored for 14 days.

3.4.5 Cage side observation

The general behavior of mice was observed during the acclimatization period. After administration of the saffron ethanolic extracts, each mouse was continuously monitored at

intervals of 30 min, 1 h, 2 h and 3 h followed by every 2 hours till 24 h and thereafter, twice daily for 14 days to detect clinical signs that included the signs of toxicity, mortality, and the latency of death. The monitored parameters included evaluation of general behavior, properties of skin and fur, eyes, changes in appearance, respiratory pattern, autonomic nervous system features such as salivation, diarrhea, and urination, central nervous system features including the morbidly and mortality and any other abnormal behavior.

3.4.6 Body weight, food, and water consumption

The body weight of mice was assessed using a sensitive balance during the acclimatization period, once before the commencement of dosing, once weekly during the dosing period and once on the day of sacrifice. Furthermore, the amount of feed and water intake were observed throughout the study period. The feed and water consumption were measured from the quantity of feed and water supplied minus the quantity remained after 24 h. The individual record on all observations was maintained for each animal.

3.4.7 Anesthesia

On the 14th day, the animals were made to fast overnight but were allowed water ad libitum. Animals were anesthetized in chloroform chamber and the blood was drawn through cardiac puncture method as described by Parasuraman et al. (2010) and collected into two tubes: one with anticoagulant, ethylene diamine tetra acetic acid (EDTA) and the other without anticoagulant.

3.4.8 Hematological analysis

The blood sample collected in EDTA containing tube was used to evaluate the hematological parameters, such as total red blood cells (RBC), hemoglobin (Hb), hematocrit

(HCT), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), total white blood cells (WBC), red blood cell distribution width (RDW), platelet (Plt), neutrophils (Nue), lymphocytes (Lym), monocytes (Mon), eosinophils (Eos) and basophils (Bas). The hematological parameters were determined using HEMAVET 950 Hematology Analyzer (Drew Scientific, Dusseldorf, Germany).

3.4.9 Biochemical analysis

The blood sample collected in plain tubes without EDTA was evaluated for biochemical analysis. The samples were allowed to clot after being centrifuged at 3000 rpm for 10 min at 4 °C to separate the serum. Afterward, the serum was transferred to Eppendorf tubes with micropipette and stored at 4 °C until assayed for biochemical evaluation. Serum biochemistry parameters, such as total cholesterol (TC) and total triglyceride (TG) were measured by Microlab 300 Biochemical Analyzer (Elitech Group, Germany) whereas, aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP) were analyzed by Fuji Biochemical Analyzer Dri-Chem 4000i (Fujifilm, Dusseldorf, Germany).

3.4.10 Relative organ weight

The mice after being painlessly sacrificed were analyzed for relative weight of liver, kidney, pancreas, and heart organs. The organs weight was recorded after being washed with normal saline and dried using blotting paper. Some of the vital organs of mouse are shown in figure 3.1. The relative weight index of each organ was calculated by the formula given below:

$$\text{Weight of organ/total weight of mice on the day of sacrifice} \times 100\% \quad (3.1)$$

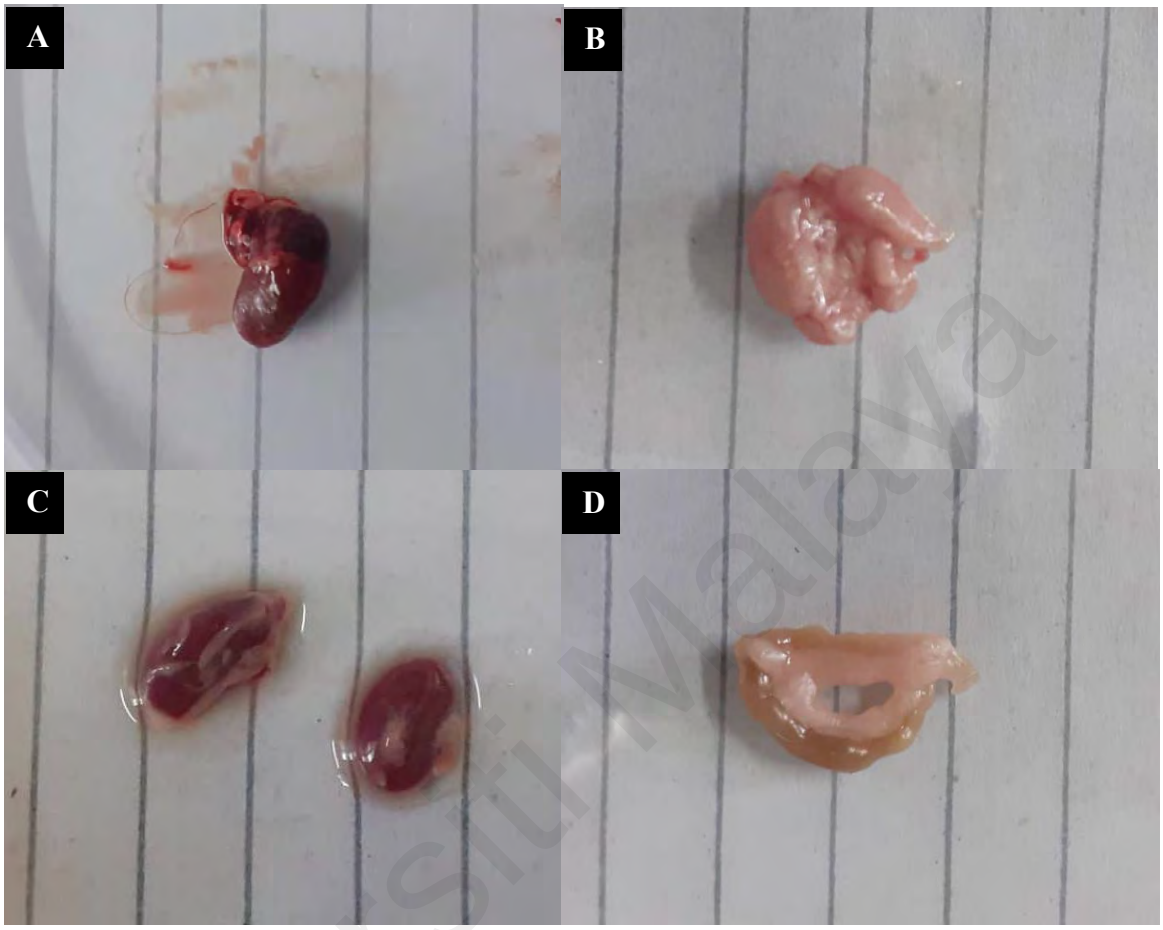


Figure 3.1: Vital organs of control group (A) heart (B) brain (C) kidneys and (D) pancreas

3.4.11 Histopathological analysis

For histological analysis, tissue samples of kidneys, heart and pancreas collected from mice, were fixed in formalin (10%). The organs were cleaved, prepared using ethanol (70%, 90% and 100%), and paraffin for embedding. Xylene was used to de-wax different sections (6 µm thick). Sections were stained by using hematoxylin-eosin (H&E) dye and tissues were analyzed under optical microscope for their general structure and degenerative changes.

3.4.12 Statistical analysis

One-way ANOVA was performed using GraphPad Prism V 6.0 (GraphPad Software Inc., San Diego, CA, USA). Dunnett comparison test was chosen as post hoc analysis method where $p \leq 0.05$ was considered to be statistically significant. The results were expressed as mean ± SEM.

3.5 Pharmacological activities of saffron

3.5.1 Analgesic, anti-inflammatory, anticoagulant and antidepressant assay

3.5.1.1 Chemicals

All the chemicals, including aspirin, diclofenac potassium, fluoxetine HCl, ethanol, and carrageenan were purchased from Sigma. All chemicals used in the experiment were analytical grade.

3.5.1.2 Standard and test drugs preparation

Ethanolic extracts of saffron were prepared as 0.08 g/mL in 10% DMSO while standard drugs were dissolved in saline water (0.01 g/mL of 0.9% NaCl) and treated orally at 1 mL/100 g of mouse body weight. One optimal dose (800 mg/kg) showing maximum effect with no lethal consequences to mice was selected in this study, as reported previously (Iranshahi et al., 2011).

3.5.1.3 Experimental animals

Adult albino mice (Swiss strain) of either sex between 30–40 g were provided by Veterinary Research Institute Peshawar and similar conditions were applied as described in section 3.4.3.

3.5.1.4 Experimental design

Mice were weighed, marked with numbers, and split into 6 groups, each containing 6 mice. All the samples were administered by oral gavage. Group 1 received saline (0.9%) and was used as a negative control. Mice in group 2 were given a dose of 10 mg/kg standard drugs (aspirin for analgesic and anticoagulant, diclofenac potassium for anti-inflammatory, and fluoxetine HCl for antidepressant assay) used as a positive control. Group 3, 4, 5, and 6 were administered with a dose of 800 mg/kg CEE, LEE, SEE, and PEE, respectively.

3.5.1.5 Hot plate analgesic test

The test first reported by Eddy & Leimbach (1953), with some modifications, was performed to measure the analgesic activity of saffron extracts. The standard drug, aspirin (10 mg/kg), and saline (1 mL/kg) were served as a positive and negative control, respectively. The parameter recorded was based on the latency time for fore- and hind-paw licking and/or jumping responses by placing the mice on the surface of a hot-plate (IITC Life Science, USA) set at a temperature of 55 ± 1 °C and initial latency time (Ti) was calculated by taking the mean of two readings. Animals with baseline latencies of <5 s or >30 s were ignored from the study. The final latency time (Tf) was noted after administration of the drug for each group at the intervals of 0.5, 1, and 2 h, with a cut off time of 30 s. The tested extracts (0.8 g/kg) were administered orally, and standard (aspirin, 10 mg/kg) was administered subcutaneously. The percentage analgesia (PA) was measured using the formula given in 3.2.

$$PA = (Tf\text{-}Ti/Ti) \times 100 \quad (3.2)$$

3.5.1.6 Carrageenan-induced paw edema test

C. sativus ethanolic extracts were examined for their anti-inflammatory activities using the carrageenan-induced paw edema test as described by Winter et al. (1962). The test is

based on the principle to assess acute anti-inflammatory activity in the hind paws of mice by developing inflammatory models with carrageenan, a sulfated polysaccharide agent that triggers inflammation. In this test, diclofenac potassium (10 mg/kg) was used as a positive control, and saline (1 mL/kg) served as a negative control to compare the anti-inflammatory effects. Before experimentation, mice were fasted for 24 h but had access to water. For edema induction, 1% λ-carrageenan (0.1 mL), prepared in 1% saline was injected into the sub planter tissue of mice left hind paw 1 h after administration of the drug. The basal volume of the paw was recorded just before and after injection of λ-carrageenan at 0, 1, 2, 3, and 4 h by digital plethysmometer (UGO Basile, 7140). For every interval, all the data were recorded in triplicate. The degree of swelling was calculated by the delta volume (A-B), where "A" shows the mean volume of the left hind paw after and "B" before the treatment of *λ*-carrageenan. The percentage edema inhibition (PEI) was measured using the formula (3.3),

$$PEI = (A\text{-}B/A) \times 100 \quad (3.3)$$

3.5.1.7 Anticoagulant test

Blood clotting activity of saffron extracts was evaluated by the capillary tube method reported by Ismail & Mirza (2015). One hour after oral administration of the dose, the mouse tail was disinfected with spirit and prickled using a lancet. The tail was pressed firmly to get a bigger drop of blood and collected in capillary tubes. The time of appearance of the blood drop on the cut tail was recorded. The tubes were wrapped and maintained at 37 ± 1 °C in a water bath. Small portions of each tube were broken at regular intervals of 10 s until fibrin thread appeared. The blood coagulation time was measured by considering the appearance of a blood drop as a starting point and thread formation as an endpoint.

3.5.1.8 Forced swimming test

The forced swim test (FST) described by Porsolt et al. (1979) was used to screen the extracts of saffron for their antidepressant activity. On day first, mice were forced to swim individually in a glass tank of 40 cm height and 18 cm diameter containing water up to 30 cm under natural light**.** The water temperature was adjusted to 25 ± 1 °C. The water level was adjusted in such a way that mice could only touch the bottom of the tank with the tip of the tail. After 15 min exposure in the tank, mice were evacuated, dried off with a paper towel, and shifted to their home cage. The next day, saline (1 mL/kg) and fluoxetine HCl (10 mg/kg) served as a negative and positive control, respectively, along with saffron extracts (800 mg/kg) were administered 30 min before the experiment. Mice were allowed to swim freely for 6 min in the tank, and swimming behaviors were recorded with a video camera. Before each test, freshwater was introduced. Animals were placed one by one, and after every 2 min, immobility time was calculated in the last 4 min of swimming practice using a stopwatch. Immobility time is the time when the mouse stopped all additional movements other than those required for survival or balancing of their body.

3.5.1.9 Statistical analysis

Data were expressed as mean along with standard deviation. Significant difference between groups was analyzed with One-way ANOVA in anticoagulant and antidepressant assays and two-way ANOVA in analgesic and anti-inflammatory assays followed by the post hoc Dunnett test. Graphs were made in GraphPad Prism 5.0 (La Jolla, CA, USA). The results were considered to be significant at $p < 0.05$.

3.5.2 Antidiabetic effect

3.5.2.1 Plant materials and chemicals

Saffron ethanolic extracts of corm, leaf, petals, and stigma extracted in chapter 3.4.2 were used in the present study. Streptozotocin (STZ) was purchased from Sigma Chemical Company (St Louis, MO, USA). Reagent kits for the determination of triacylglycerol (TG), total cholesterol (TC), and high-density lipoprotein cholesterol (HDL-C) were from Kexin Biotechnology Institute (Shanghai, China). All other chemicals used were of analytical grade.

3.5.2.2 Experimental animals

Adult albino male mice (Swiss strain) were obtained from VRI Peshawar, and same conditions were applied as described in section 3.4.3.

3.5.2.3 Induction of type 2 diabetes

On the first day of study, freshly prepared streptozotocin in cold sterile normal saline was given intraperitoneal (i.p) injection at a dose of 40 mg/kg to overnight fasted mice for 3 consecutive days. The normal control group were fed a basic diet and water, whereas the experimental group was fed a high fat diet for a period of 2 weeks. The composition of the diet fed to the mice is shown in Table 3.2. The experimental mice were kept on 5% glucose solution bottles in their cages instead of simple water to prevent excessive hypoglycemia. The blood glucose level was recorded using Accu-Chek, Glucometer by puncturing tail tip of mice. The values of glucose were recorded in mg/dl of blood. The animals developed diabetes after one week of injection. The animals with blood glucose range of 250–300 mg/dl were used for the experiment.

Table 3.2: Composition of high-fat diet fed to diabetic mice

Ingredients	**%/100 g**
Fat	42
Protein	15
Carbohydrate	43
Ingredients	**g/100 g**
Powdered rodent feed	68.0
Milk powder	20.0
Maize oil	6.0
Ghee	6.0
Total energy (kcal/100 g)	414.0

3.5.2.4 Experimental Design

All the mice were divided into six groups according to their weights and blood glucose levels to make the average weights and blood glucose levels similar among the groups.

Group 1: Normal control treated with distilled water

Group 2: Diabetic control received only distilled water

Group 3: Diabetic mice treated with CEE (40 mg/kg/day) for 21 days

Group 4: Diabetic mice treated with LEE (40 mg/kg/day) for 21 days

Group 5: Diabetic mice treated with PEE (40 mg/kg/day) for 21 days

Group 6: Diabetic mice treated with SEE (40 mg/kg/day) for 21 days

The daily dosage of saffron ethanolic extracts used in this experiment was estimated from a published literature. All mice continued to get ad libitum water and normal pellet diet. Saffron ethanolic extracts were prepared just before the oral administration with distilled water. The fasting blood glucose levels of the mice were recorded every week during the experiment period.

3.5.2.5 Glucose tolerance test in diabetic animals

Oral glucose tolerance test was performed following the method described by Bari et al. (2020) with slight modifications. Prior to the experiment, all mice were kept fasting for 15 h. Group 1 and 2 received 5% DMSO at a dose of 1 ml/kg to mice. Group 3, 4, 5 and 6 mice were orally administered with 50 mg/kg of CEE, LEE, PEE and SEE respectively, followed by oral treatment with glucose solution (0.5 g/kg BW) after 30 min of extract administration. Blood glucose level was measured using portable glucometer from tail veins before and 30 min, 60 min and 120 min after glucose intake.

3.5.2.6 Assessment of antidiabetic activity

Mice were fed with usual diet (rodent chow and water ad libitum) and received their respective treatments for 21 days. Blood glucose level of fasted animals (15 h) was measured on 1st, 7^{th},14^{th} and 21^{st} day from tail veins by glucometer.

3.5.2.7 Collection of blood and tissue samples

At the end of the experimental period, animals fasted for 12 h prior were anesthetized under chloroform chamber for 2-5 min and sacrificed. The blood was drawn by the cardiac puncture method in anticoagulant-free tubes and then centrifuged at 3000 rpm for 10 min to separate the serum from cellular contents and stored at −20 °C to measure biochemical parameters. Kidneys, heart, and pancreas from each animal were excised and fixed in 10% formalin for histology.

3.5.2.8 Measurement of serum lipid profile

The concentrations of TG, TC, HDL cholesterol, and LDL cholesterol in serum were estimated by using diagnostic kits on an automatic analyzer (Abbott, model Alcyon 300, USA). Results were expressed as mg/dL.

3.5.2.9 Measurement of liver functions

The concentrations of liver enzymes such as ALT, AST, and ALP, was evaluated to determine the enzymatic activities of the liver of the normal and the experimental groups by commercially available standard diagnostic kits according to the manufacturer's protocol.

3.5.2.10 Measurement of kidney functions

The indicator of renal functions such as serum creatinine was detected using colorimetric kinetic method, whereas blood urea nitrogen (BUN) and uric acid (UA) were analysed by enzymatic calorimetric method according to the manufacturer's protocol.

3.5.2.11 Histopathological examination

The organs were cleaved, prepared using ethanol (70%, 90% and 100%), and paraffin for embedding. Xylene was used to de-wax different sections (6 µm thick). Sections were stained by using H&E dye and tissues were analyzed under light microscope (DIALUX 20 EB) at 10X magnification for their general structure and degenerative changes. Slides of all groups were studied and photographed.

3.5.2.12Statistical analysis

One-way ANOVA was performed using GraphPad Prism V 6.0. Dunnett comparison test was chosen as post hoc analysis method where $p \leq 0.05$ was considered to be statistically significant. The results were expressed as mean ± SEM.

RESULTS

4.1 Micropropagation

4.1.1 Seed sterilization

Among all the sterilization protocols used, sterilization of corms for 2-3 min with 70% ethyl alcohol, washing with Tween 20 for 30 min followed by 40% Clorox for 15 min and 0.20% $HgCl_2$ for 10 min showed the highest percentage for aseptic cultures (93%) and survival rate (93%) as shown in table 4.1. Higher concentration of $HgCl_2$ (0.30%) and Clorox (50-60%) ensured maximum aseptic cultures (96%) but decreased explant viability (70%) and resulted in necrosis.

4.1.2 Bud sprouting

Bud sprouting efficiency of saffron per corm was higher in MS medium as compared to Gamborg medium under all kinds of auxins (NAA, IAA) and cytokinin (Kn, BAP). Highest bud sprouting response (100%) and number of sprouted corms (4.90) were achieved when corms were cultured on MS medium fortified with IAA (1 mg/l) in combination with BAP (2 mg/l) as shown in table 4.2. Similar results with no significant difference were observed with IAA (1 mg/l) and BAP (4 mg/l), whereas corms cultured on MS medium containing IAA (2 mg/l) and BAP (2 mg/l) showed lowest sprouting response (40%) and sprouted number (1.40). Bud sprouting on MS with IAA (1 mg/l) and BAP (4 mg/l) is shown in figure 4.1(B). On the other hand, Gamborg medium gave highest bud sprouting response and sprouting number of 90% and 3.70 on IAA (1 mg/l) plus BAP (4 mg/l), respectively. Absence of PGRs in the medium showed the least sprouting percentage (40%) and sprouted corms (0.90) followed by medium containing NAA (2 mg/l) in combination with Kn (2 mg/l) as shown in table 4.2.

4.1.3 Shoot primordia

Corms that showed 100% success in IAA (1 mg/l) and BAP (2 mg/l) containing medium were transferred to MS medium containing IAA (1, 2 mg/l) plus different concentration of BAP (2, 4, 6 mg/l) or Kn (2, 4, 6 mg/l). After 5 weeks, direct multiple shoot primordia in the form of bunches of shoots appeared at the base of sprouted buds as shown in figure 4.1(C). Some of the single initiated shoots persisted throughout the growth period and turned yellow after reaching a certain length as shown in figure 4.1(D). Almost all conditions inducted shoot primordia but showed varied response as illustrated in table 4.3. The highest number of shoot primordia (5.60) was achieved in MS fortified with IAA (1 mg/l) and BAP (4 mg/l). However, average length of shoot primordia was higher (2.33 cm) in MS medium containing IAA (1 mg/l) and BAP (6 mg/l). Inclusion of Kn in the medium showed poor response towards induction of multiple shoot primordia. Whereas least number of multiple shoot primordia (1.20) was attained in MS basal medium.

4.1.4 Multiple shoot primordia

Further sub culturing of shoot primordia on MS medium containing IAA (0.5 and 1 mg/l) in combination with BAP (2, 4 and 6 mg/l) or Kn (2, 4 and 6 mg/l) resulted in multiple shoot proliferation as shown in Fig. 4.1(E-F). In the present study, shoot multiplication increased with an increase in concentration of BAP from 2-6 mg/l in combination with IAA (0.5 mg/l) as shown in table 4.4. Among different concentration of BAP and Kn used, MS supplemented with IAA (0.5 mg/l) and BAP (6 mg/l) was most superior to enhance shoot number (6.40) and shoot length (4.38 cm) followed by combine treatment of IAA (0.5 mg/l) and BAP (4 mg/l), where the mean shoot number and shoot length was found to be 5.06 and 2.93 cm per culture, respectively. On the other hand, Kn was only effective at a concentration of 6 mg/l in combination with IAA (0.5 mg/l) giving a mean shoot number of 4.13 per culture.

4.1.5 Cormlet production

Development of daughter corms initiated after 3 to 4 weeks of culture in the form of swelling at the base of the shoots as shown in Fig. 4.1(G-H). Different concentration of sucrose and GA_3 showed significant differences in their cormlet number and cormlet weight when added to MS medium supplemented with IAA (0.5 mg/l) as depicted in table 4.5. Significantly, maximal cormlet number (4.33) and cormlet weight (0.58 g) were obtained in medium fortified with GA_3 (4 mg/l) and 4% sucrose. MS medium with no PGR and sucrose was ineffective for inducing cormlet formation. Higher concentration of GA_3 showed significant effect on cormlet proliferation and weight with no significant effect on root proliferation and length. According to data, media with 2% sucrose and GA_3 (2 mg/l) gave highest root number (5.2) and root length (13.10 cm) per root formed explants. Figure 4.1(I) shows cormlet with the rooting at the base of the mother corm.

Table 4.1: Effect of sterilization protocol on aseptic cultures and percent survival rate of corm explants

S/No.	Sterilization Protocol		Aseptic explants (%)	Explants survived (%)
	Chemicals	Time		
1.	15% H_2O_2 70% ethyl alcohol 40% Clorox	10 min 2-3 min 15 min	40.00^{d}	26.67^{d}
2.	25% H_2O_2 70% ethyl alcohol 40% Clorox	10 min 2-3 min 15 min	53.33^{cd}	40.00^{cd}
3.	70% ethyl alcohol 40% Clorox	2-3 min 15 min	56.67^{bcd}	53.33^{bc}
4.	70% ethyl alcohol 50% Clorox	2-3 min 15 min	73.33^{abc}	66.67^{b}
5.	70% ethyl alcohol 60% Clorox	2-3 min 15 min	80.00^{ab}	60.00^{bc}
6.	70% ethyl alcohol 40% Clorox 0.10% $HgCl_2$	2-3 min 15 min 10 min	66.67^{bcd}	66.67^{b}
7.	70% ethyl alcohol 40% Clorox 0.20% $HgCl_2$	2-3 min 15 min 10 min	93.33^{a}	93.33^{a}
8.	70% ethyl alcohol 40% Clorox 0.30% $HgCl_2$	2-3 min 15 min 10 min	96.67^{a}	70.00^{ab}

n = 30. Mean values with different letters within the same column differ significantly from each other at $p \leq 0.05$

Table 4.2: Sprouting response and mean number of sprouted corms per explant in MS and Gamborg medium with different concentration of auxin (NAA and IAA) and cytokinin (IAA and BAP

PGR (mg/l)				MS		Gamborg	
NAA	**Kn**	**IAA**	**BAP**	**Sprouting rate (%)**	**Mean no. of sprouted corms**	**Sprouting rate (%)**	**Mean no. of sprouted corms**
0	**0**	**0**	**0**	46^{cd}	1.06 ± 0.22^{g}	36^{f}	0.76 ± 0.19^{h}
1	**1**			73^{b}	2.36 ± 0.27^{cde}	50^{def}	1.46 ± 0.28^{fgh}
1	**2**			76^{ab}	3.40 ± 0.37^{b}	63^{bcde}	2.36 ± 0.35^{cdef}
1	**4**			83^{ab}	3.06 ± 0.29^{bc}	86^{ab}	2.70 ± 0.23^{bcd}
1	**6**			80^{ab}	3.20 ± 0.34^{bc}	90^{ab}	3.36 ± 0.25^{ab}
2	**1**			46^{cd}	1.43 ± 0.29^{fg}	56^{cdef}	1.76 ± 0.31^{efg}
2	**2**			63^{bcd}	1.96 ± 0.29^{defg}	40^{ef}	1.23 ± 0.29^{gh}
2	**4**			66^{bc}	1.96 ± 0.27^{defg}	46^{def}	1.70 ± 0.35^{efg}
2	**6**			70^{bc}	2.26 ± 0.32^{cdef}	70^{abcd}	2.03 ± 0.26^{defg}
		1	**1**	83^{ab}	2.76 ± 0.26^{bcd}	86^{ab}	3.20 ± 0.35^{cdef}
		1	**2**	100^{a}	4.90 ± 0.21^{a}	76^{abc}	3.83 ± 0.25^{abc}
		1	**4**	100^{a}	4.40 ± 0.20^{a}	93^{a}	2.40 ± 0.30^{a}
		1	**6**	80^{ab}	3.40 ± 035^{b}	70^{abcd}	1.53 ± 0.27^{cde}
		2	**1**	46^{cd}	1.46 ± 0.30^{efg}	56^{cdef}	1.36 ± 0.21^{efgh}
		2	**2**	40^{d}	1.40 ± 0.32^{fg}	63^{bcde}	2.33 ± 0.24^{gh}
		2	**4**	86^{ab}	3.03 ± 0.26^{bc}	76^{abc}	2.40 ± 0.50^{cdef}
		2	**6**	73^{b}	2.70 ± 0.32^{bcd}	83^{ab}	2.50 ± 0.40^{cde}

n = 30. Each value is represented as mean ± SD. Mean values with different letters within the same column differ significantly from each other at $p\leq0.05$

Table 4.3: Induction of shoot primordia in IAA and Kn or BAP supplemented medium

PGR (mg/l)			Mean no. of shoot primordia	Mean length of shoot primordia (cm)
IAA	Kn	BAP		
0	**0**	**0**	1.20±0.29^{c}	0.59±0.15^{cd}
1	**2**		2.00±0.30^{bc}	0.67±0.11^{cd}
1	**4**		3.10±0.35^{b}	1.02±0.11^{cd}
1	**6**		2.50±0.43^{bc}	0.69±0.12^{cd}
2	**2**		2.40±0.40^{bc}	0.66±0.11^{cd}
2	**4**		2.10±0.31^{bc}	0.54±0.09^{d}
2	**6**		2.30±0.37^{bc}	0.67±0.11^{cd}
1		**2**	3.20±0.49^{b}	1.08±0.21^{c}
1		**4**	5.60±1.06^{a}	1.85±0.17^{b}
1		**6**	3.50±0.27^{b}	2.33±0.29^{a}
2		**2**	2.70±0.42^{bc}	1.12±0.22^{c}
2		**4**	3.10±0.43^{b}	1.02±0.12^{cd}
2		**6**	2.80±0.15^{b}	0.93±0.14^{cd}

n = 15. Each value is represented as mean ± SD. Mean values with different letters within the same column differ significantly from each other at $p \leq 0$

Table 4.4: Induction of multiple shoot proliferation in IAA and Kn or BAP supplemented medium

PGR (mg/l)			Mean no. of shoot	Mean length of shoot (cm)
IAA	Kn	BAP		
0	0	0	1.93±0.18^{fg}	1.20±0.08^{g}
0.5	2		0.83±0.13^{h}	0.68±0.10^{h}
0.5	4		3.56±0.24^{c}	1.81±0.12^{ef}
0.5	6		4.13±0.22^{c}	1.80±0.12^{ef}
1	2		1.60±0.15^{g}	1.32±0.11^{g}
1	4		2.96±0.18^{d}	2.65±0.20^{bc}
1	6		2.30±0.16^{ef}	1.47±0.11^{fg}
0.5		2	2.93±0.17^{d}	2.83±0.18^{bc}
0.5		4	5.06±0.26^{b}	2.93±0.17^{b}
0.5		6	6.40±0.26^{a}	4.38±0.14^{a}
1		2	2.66±0.19^{de}	2.16±0.19^{de}
1		4	3.70±0.18^{c}	2.43±0.18^{cd}
1		6	3.93±0.20^{c}	2.66±0.14^{bc}

n = 15. Each value is represented as mean ± SD. Mean values with different letters within the same column differ significantly from each other at $p \leq 0.05$

Table 4.5: Rooting and cormlet formation in IAA and GA_3 supplemented medium

MS media + 1 IAA	Cormlet number	Cormlet weight (g)	Root number	Root length (cm)
Control	NR	NR	NR	NR
3% sucrose + 1 GA3	1.10 ± 0.28^{d}	0.30 ± 0.07^{b}	2.20 ± 0.13^{e}	2.06 ± 0.22^{d}
3% sucrose + 2 GA3	1.40 ± 0.40^{cd}	0.24 ± 0.06^{b}	2.60 ± 0.26^{de}	2.84 ± 0.33^{d}
3% sucrose + 4 GA3	2.30 ± 0.39^{bc}	0.38 ± 0.05^{b}	3.40 ± 0.16^{bcd}	2.36 ± 0.36^{d}
4% sucrose + 1 GA3	1.90 ± 0.37^{cd}	0.37 ± 0.07^{b}	2.80 ± 0.24^{cde}	2.88 ± 0.33^{d}
4% sucrose + 2 GA3	1.50 ± 0.31^{cd}	0.34 ± 0.06^{b}	3.20 ± 0.32^{bcd}	2.62 ± 0.42^{d}
4% sucrose + 4 GA3	3.40 ± 0.30^{a}	0.58 ± 0.09^{a}	3.80 ± 0.38^{b}	3.76 ± 0.30^{d}
5% sucrose + 1 GA3	1.10 ± 0.23^{d}	0.39 ± 0.07^{b}	3.60 ± 0.26^{bc}	6.20 ± 0.64^{c}
5% sucrose + 2 GA3	2.20 ± 0.36^{bc}	0.38 ± 0.04^{b}	5.20 ± 0.38^{a}	13.10 ± 1.89^{a}
5% sucrose + 4 GA3	3.10 ± 0.23^{ab}	0.32 ± 0.01^{b}	4.80 ± 0.32^{a}	9.22 ± 0.48^{b}

n = 10. Each value is represented as mean ± SD. Mean values with different letters within the same column differ significantly from each other at $p\leq0.05$

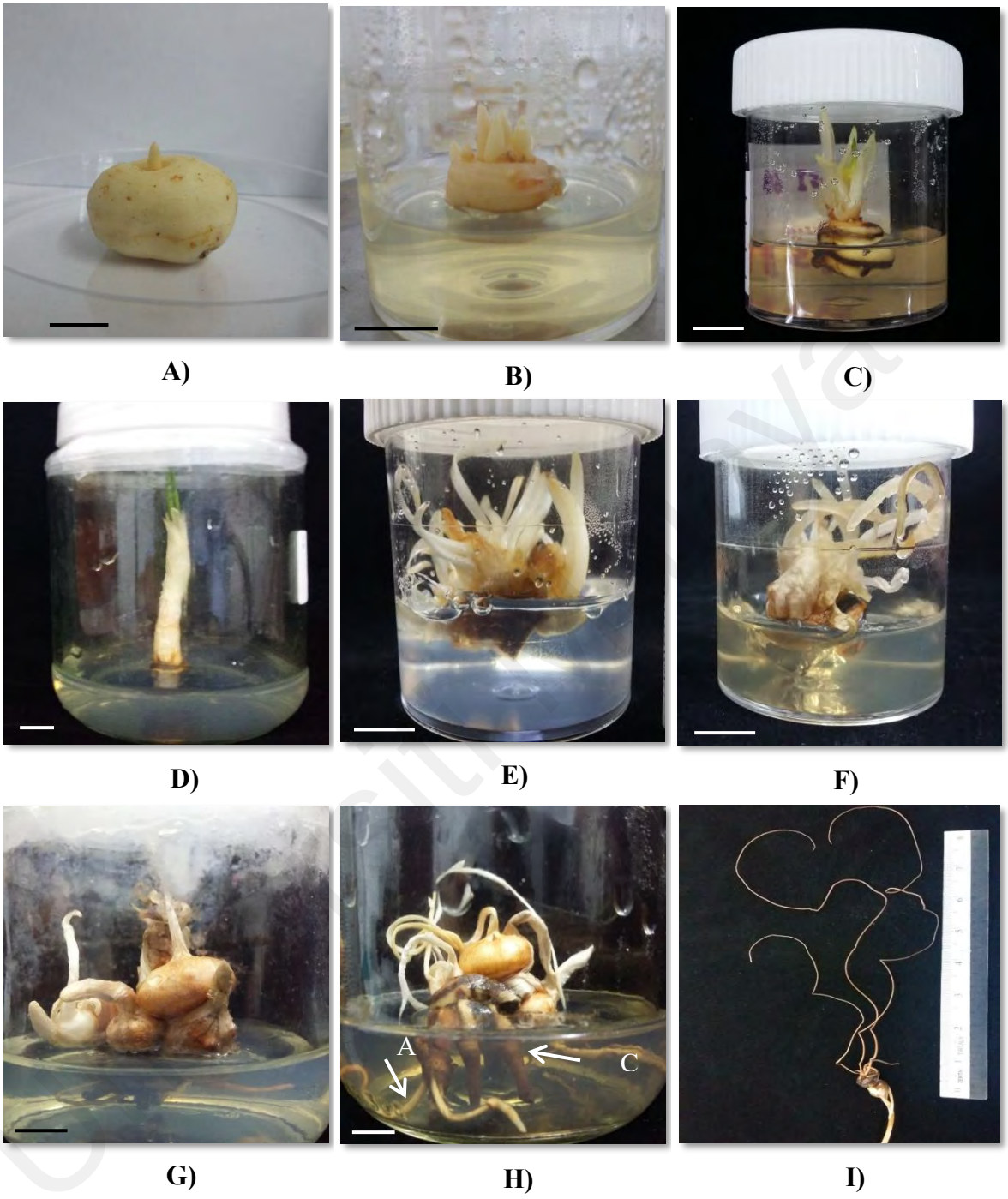


Figure 4.1: *In vitro* shoot multiplication & cormlet formation in saffron (A) corm used as explant. (B) Buds sprouting on MS with IAA (1 mg/l) and BAP (4 mg/l). (C) Shoot primordia on MS with IAA (1 mg/l) and BAP (4 mg/l). (D) Sprouted bud emerged to form a single shoot on MS with IAA (2 mg/l) and BAP (2 mg/l). (E-F) Multiple shoot primordia. (G) Cormlets produced in MS with IAA (1 mg/l), 4% sucrose and GA_3 (4 mg/l). (H) Cormlet with the rooting (contractile root and absorbing root) at the base of mother corm. (I) AR of mother corm. Bar line = 1.0 cm

4.2 Agro-Environmental factors affecting saffron

According to our results, using mother corms of 10-15 g weight grown at incubation temperature of 18 and 24 °C under black soil containing chicken fertilizer or manure at a planting depth of 8-16 cm influenced reproductive phase of saffron under both years. The principal growth stages of saffron are given in the figure 4.2.

Figure 4.2: The principal growth stages of saffron A) mother corm B) sprouting of mother corm C) leaf development D) flower formation

4.2.1 Effect of single factor

The effect of temperature, mother corm size, fertilizer, planting depth and year on flower and daughter corms indices of saffron is given in table 4.6. According to the results, except planting depth, all other factors had a significant effect on floral and daughter corm related attributes. The highest flower fresh weight (FFW), flower dry weight (FDW), stigma fresh weight (SFW), stigma dry weight (SDW), daughter corm number (DCN) and daughter corm weight (DCW) were attained when corms were grown at 18 °C. On the contrary, by increasing the temperature up to 24 °C, a significant reduction in the growth parameters was observed. Similarly, maximum amount of flower and daughter corm yield for all variables were obtained significantly when mother corms were bigger in size compared to smaller mother corms. Regarding type of fertilizer, application of cattle manure to soil showed a significant increase in all the reproductive and vegetative parameters of saffron when compared with the soil containing chicken fertilizer. On the other hand, planting depth had no significant effect on all the variables related to flowers (FFW, FDW, SFW and SDW) and daughter corms (DCN and DCW). The data given in table 4.6 also showed that saffron growth indices were significantly affected by planting seasons. Except for DCW, other growth indices such as FFW, FDW, SFW, SDW and DCN after the end of the 1st year were significantly higher than 2nd year experiment. Whereas DCW was significantly higher after the 2nd harvesting season as compared to year 1.

Table 4.6: Summary of the effects of temperature, mother corm size, type of fertilizer, planting depth and harvesting year on saffron growth indices

Experimental treatments	Reproductive parameters (g)				Vegetative parameters	
	Flower fresh weight	Flower dry weight	Stigma fresh weight	Stigma dry weight	Daughter corm number	Daughter corm weight (g)
Temperature						
18 °C	156.48[a]	30.29[a]	27.70[a]	6.01[a]	4.35[a]	4.13[a]
24 °C	145.10[b]	27.60[b]	26.18[b]	5.69[b]	3.89[b]	3.73[b]
Corm weight						
10 g	139.22[b]	26.93[b]	25.29[b]	5.48[b]	3.87[b]	4.04[a]
15 g	162.36[a]	30.96[a]	28.59[a]	6.21[a]	4.38[a]	3.82[b]
Type of fertilizer						
Chicken fertilizer	143.98[b]	27.53[b]	25.95[b]	5.64[b]	3.88[b]	3.77[b]
Cow manure	157.60[a]	30.36[a]	27.94[a]	6.05[a]	4.37[a]	4.09[a]
Planting depth						
8 cm	152.07[a]	29.21[a]	27.17[a]	5.88[a]	4.17[a]	3.95[a]
16 cm	149.51[a]	28.68[a]	26.72[a]	5.82[a]	4.08[a]	3.91[a]
Harvesting year						
Year 1	155.40[a]	29.42[a]	27.91[a]	6.02[a]	4.39[a]	3.81[b]
Year 2	146.17[b]	28.47[b]	25.97[b]	5.68[b]	3.85[b]	4.05[a]

Mean values with different letters within the same column differ significantly from each other at $p \leq 0.05$.

4.2.2 Interaction effect of temperature and harvesting year

Table 4.7 shows the growth parameters of saffron in response to the interaction effect of temperature and harvesting year. The results illustrated that all single factors of temperature and harvesting year as well as their interaction had no significant effect on the floral and vegetative yield components of saffron. However, lower temperature under both years was more suitable for higher floral yield and daughter corms formation.

4.2.3 Interaction effect of corm weight and harvesting year

The data given in Table 4.8 demonstrated that saffron growth indices were significantly affected by interaction between year and mother com size. Mother corm class of 15 g showed significantly higher FFW of 166 and 157.85 g/plant as compared to mother corms of 10 g (143.95 and 134.50 g/plant) during the 1st and 2nd year, respectively. Similarly, highest FDW (31.60 and 30.33 g/plant) was achieved significantly with bigger corms than smaller corms (27.25 and 26.22 g/plant) in the 1st and 2nd year of experiment. Regarding stigma indices, SFW reached the highest amount (29.80 and 27.39 g/plant) significantly with bigger corms compared with the smaller corms (26.04 and 24.56 g/plant) in the two growing seasons. Likewise, in the 1st and 2nd year of saffron harvesting season, bigger corms displayed significantly higher SDW (6.43 and 6.0 g/plant) than the smaller corms (5.61 and 5.37 g/plant). Our results illustrated that the number of daughter corms derived from mother corm weighing more than 15 g was higher (4.67 and 4.09 per corm) than the daughter corms produced from smaller corms (4.13 and 3.63 per corm) after the completion of 1st and 2nd year. In the 1st year, daughter corms formed from mother corms weighing 10 g were significantly bigger in size (3.93 g) than corms formed from 15 g (3.69 g). However, no significant difference in weight of daughter corms produced from mother corms of both classes was reported during the 2nd year.

Table 4.7: Interaction effect of temperature and harvesting year on saffron growth indices

Year	Temperature	FFW (g)	FDW (g)	SFW (g)	SDW (g)	DCN	DCW (g)
Year 1	18 °C	160.18[a]	31.02[a]	28.74[a]	6.20[a]	4.56[a]	4.03[a]
	24 °C	150.64[a]	27.82[a]	27.10[a]	5.85[a]	4.23[a]	3.59[a]
Year 2	18 °C	152.78[a]	29.56[a]	26.68[a]	5.82[a]	4.16[a]	4.24[a]
	24 °C	139.58[a]	27.38[a]	25.27[a]	5.55[a]	3.56[a]	3.88[a]

Mean values with different letters within the same column differ significantly from each other at $p \leq 0.05$

Table 4.8: Interaction effect of corm size and harvesting year on saffron growth indices

Year	Corm size	FFW (g)	FDW (g)	SFW (g)	SDW (g)	DCN	DCW (g)
Year 1	10 g	143.95[b]	27.25[b]	26.04[ab]	5.61[b]	4.13[ab]	3.93[a]
	15 g	166.87[a]	31.60[a]	29.80[a]	6.43[a]	4.67[a]	3.69[b]
Year 2	10 g	134.50[b]	26.62[b]	24.56[b]	5.37[b]	3.63[c]	4.16[a]
	15 g	157.85[a]	30.33[a]	27.39[a]	6.00[a]	4.09[b]	3.96[a]

Mean values with different letters within the same column differ significantly from each other at $p \leq 0.05$

4.2.4 Interaction effect of type of fertilizer and harvesting year

The interaction of type of fertilizer and harvesting year on saffron reproductive and vegetative indices are depicted in table 4.9. The data showed that soil amendment such as fertilizer and manure significantly influenced floral fresh weight during the two growing seasons. FFW significantly reached the highest amount of 160.52 and 154.69 g/plant in saffron plants derived from mother corms grown in soil containing cattle manure than fertilizer (150.30 and 137.66 g/plant) in the 1st and 2nd year of experiment. As expected, the least amount of FDW (30.69 and 30.03 g/plant) was obtained by the corms grown in soil with manure compared to fertilizer (28.15 and 26.91 g/plant) at the end of year 1 and 2. Likewise, SFW and SDW were significantly higher in soil with manure application in the 2nd year. However, no significant difference in SFW and SDW of plants cultivated in either type of fertilizers was observed during the 1st year of growing season. In addition, daughter corm number in 2nd year and daughter corm weight in 1st year were significantly higher with manure application, whereas daughter corm number in 1st year and daughter corm weight produced from mother corms in 2nd year showed no significant result with the application of soil amendments.

4.2.5 Interaction effect of planting depth and harvesting year

The interaction of planting depth and harvesting year on saffron growth parameters are illustrated in table 4.10. Except for FDW and DCW, other growth indices such as FFW, SFW, SDW and DCN after the end of 1st year were significantly higher than 2nd year irrespective of planting depth. On the other hand, DCW was significantly higher after 2nd harvesting season as compared to 1st year irrespective of planting depth, whereas no significant difference with respect to planting depth as well as year of cultivation was observed for FDW.

Table 4.9: Interaction effect of type of fertilizer and harvesting year on saffron growth indices

Year	Type of fertilizer	FFW (g)	FDW (g)	SFW (g)	SDW (g)	DCN	DCW (g)
Year 1	Fertilizer	150.30^{b}	28.15^{ab}	27.04^{a}	5.83^{ab}	4.22^{a}	3.65^{b}
	Manure	160.52^{a}	30.69^{a}	28.80^{a}	6.22^{a}	4.58^{a}	3.97^{a}
Year 2	Fertilizer	137.66^{c}	26.91^{b}	24.87^{b}	5.47^{b}	3.55^{b}	3.90^{a}
	Manure	154.69^{ab}	30.03^{a}	27.08^{a}	5.90^{a}	4.17^{a}	4.22^{a}

Mean values with different letters within the same column differ significantly from each other at $p \leq 0.05$

Table 4.10: Interaction effect of planting depth and harvesting year on saffron growth indices

Year	Planting depth	FFW (g)	FDW (g)	SFW (g)	SDW (g)	DCN	DCW (g)
Year 1	8 cm	157.23^{a}	29.87^{a}	28.26^{a}	6.09^{a}	4.47^{a}	3.84^{b}
	16 cm	153.59^{a}	28.97^{a}	27.58^{a}	5.95^{a}	4.33^{a}	3.78^{b}
Year 2	8 cm	146.91^{b}	28.55^{a}	26.08^{ab}	5.67^{b}	3.88^{b}	4.07^{a}
	16 cm	145.45^{b}	28.39^{a}	25.87^{b}	5.69^{b}	3.84^{b}	4.05^{a}

Mean values with different letters within the same column differ significantly from each other at $p \leq 0.05$

4.2.6 Interaction effect of temperature and mother corm weight

Table 4.11 shows the reproductive and vegetative indices of saffron obtained for the interaction between temperature and mother corm weight. Except for daughter corm weight, the mean value of all other growth parameters of saffron were not significantly different showing no interaction between temperature and weight of mother corm. However, the weight of daughter corm obtained from bigger mother corms were significantly higher at low temperature compared with high temperature.

4.2.7 Interaction effect of temperature and type of fertilizer

Flowering and daughter corms formation's data illustrated in table 4.12 demonstrated that all single factors of temperature and type of fertilizer as well as their interaction had no significant effect on the six variables studied. However, the response of floral and vegetative attributes was more favorable under manure rich soil grown at low temperature.

4.2.8 Interaction effect of temperature and planting depth

The interaction between temperature and planting depth on saffron production is given in table 4.13. The data revealed that all saffron growth attributes were not significantly different irrespective of temperature and planting depth. However, the response of reproductive attributes was more prominent at low temperature and deep planting depth.

Table 4.11: Interaction effect of temperature and mother corm size on saffron growth indices

Temperature	Corm size	FFW (g)	FDW (g)	SFW (g)	SDW (g)	DCN	DCW (g)
18 C	10 g	142.12[a]	27.77[a]	25.65[a]	5.57[a]	4.17[a]	4.11[a]
	15 g	170.84[a]	32.82[a]	29.77[a]	6.44[a]	4.55[a]	4.17[a]
24 C	10 g	136.33[a]	26.09[a]	24.95[a]	5.41[a]	3.58[a]	3.98[ab]
	15 g	153.88[a]	29.11[a]	27.42[a]	5.99[a]	4.22[a]	3.49[b]

Mean values with different letters within the same column differ significantly from each other at $p \leq 0.05$.

Table 4.12: Interaction effect of temperature and type of fertilizer on saffron growth indices

Temperature	Type of fertilizer	FFW (g)	FDW (g)	SFW (g)	SDW (g)	DCN	DCW (g)
18 C	Fertilizer	149.98[a]	28.90[a]	26.73[a]	5.81[a]	4.08[a]	3.93[a]
	Manure	162.98[a]	31.68[a]	28.69[a]	6.20[a]	4.64[a]	4.35[a]
24 C	Fertilizer	137.98[a]	26.16[a]	25.18[a]	5.49[a]	3.69[a]	3.62[a]
	Manure	152.23[a]	29.04[a]	27.19[a]	5.91[a]	4.11[a]	3.85[a]

Mean values with different letters within the same column differ significantly from each other at $p \leq 0.05$

Table 4.13: Interaction effect of temperature and planting depth on saffron growth indices

Temperature	Planting depth	FFW (g)	FDW (g)	SFW (g)	SDW (g)	DCN	DCW (g)
18 C	8 cm	155.47[a]	30.15[a]	27.67[a]	5.99[a]	4.44[a]	4.14[a]
	16 cm	157.49[a]	30.43[a]	27.75[a]	6.02[a]	4.28[a]	4.08[a]
24 C	8 cm	148.67[a]	28.27[a]	26.67[a]	5.78[a]	3.91[a]	3.77[a]
	16 cm	141.55[a]	26.94[a]	25.70[a]	5.62[a]	3.89[a]	3.70[a]

Mean values with different letters within same column differ significantly from at $p \leq 0.05$

4.2.9 Interaction effect of mother corm weight and type of fertilizer

Saffron flower formation and yield components response to the interaction of mother corm weight and type of fertilizer is presented in table 4.14. It was shown that except daughter corm weight derived from bigger corms in manure rich soil, no significant difference on all saffron growth attributes was found in the single factor i.e temperature and planting depth as well as the interaction of temperature x planting depth. However, the interaction between bigger corms and cattle manure was more suitable for higher floral and daughter corms formation.

4.2.10 Interaction effect of mother corm weight and planting depth

The floral and vegetative indices of saffron obtained for the interaction between mother corm weight and planting depth is shown in table 4.15. The results demonstrated that mother corm weight and planting depth as a single factor as well as their interaction had no significant effect on all the six variables of saffron. However, the response of floral yield components to bigger corms grown at low depth was more remarkable.

4.2.11 Interaction effect of type of fertilizer and planting depth

Likewise, data shown in table 4.16 regarding flower and daughter corms formation with respect to interaction of type of fertilizer and planting depth demonstrated that all single factors as well as their interaction had no significant effect on saffron yield.

Table 4.14: Interaction effect of corm size and type of fertilizer on saffron growth indices

Corm size	Type of fertilizer	FFW (g)	FDW (g)	SFW (g)	SDW (g)	DCN	DCW (g)
10 g	Fertilizer	131.74[a]	25.47[a]	24.18[a]	5.26[a]	3.67[a]	3.99[a]
	Manure	146.71[a]	28.39[a]	26.42[a]	5.72[a]	4.08[a]	4.10[a]
15 g	Fertilizer	156.22[a]	29.59[a]	27.72[a]	6.04[a]	4.09[a]	3.56[b]
	Manure	168.50[a]	32.33[a]	29.47[a]	6.39[a]	4.67[a]	4.10[a]

Mean values with different letters within the same column differ significantly from each other at $p \leq 0.05$

Table 4.15: Interaction effect of corm size and planting depth on saffron growth indices

Corm size	Planting depth	FFW (g)	FDW (g)	SFW (g)	SDW (g)	DCN	DCW (g)
10 g	8 cm	139.24[a]	27.15[a]	25.33[a]	5.46[a]	3.98[a]	4.08[a]
	16 cm	139.21[a]	26.71[a]	25.26[a]	5.52[a]	3.77[a]	4.01[a]
15 g	8 cm	164.90[a]	31.27[a]	29.01[a]	6.31[a]	4.36[a]	3.83[a]
	16 cm	159.82[a]	30.65[a]	28.18[a]	6.12[a]	4.41[a]	3.79[a]

Mean values with different letters within the same column differ significantly from each other at $p \leq 0.05$

Table 4.16: Interaction effect of type of fertilizer and planting depth on saffron growth indices

Type of fertilizer	Planting depth	FFW (g)	FDW (g)	SFW (g)	SDW (g)	DCN	DCW (g)
Fertilizer	8 cm	147.03[a]	28.07[a]	26.36[a]	5.71[a]	3.92[a]	3.78[a]
	16 cm	157.11[a]	30.35[a]	27.98[a]	6.06[a]	4.42[a]	4.13[a]
Manure	8 cm	140.93[a]	26.99[a]	25.54[a]	5.59[a]	3.84[a]	3.77[a]
	16 cm	158.10[a]	30.37[a]	27.91[a]	6.06[a]	4.33[a]	4.07[a]

Mean values with different letters within same column differ significantly from at $p \leq 0.05$

4.3 Effect of gamma irradiation on saffron production

4.3.1 Gamma radiation effect on vegetative parameters

The effect of gamma irradiation on saffron vegetative attributes showed significant differences with respect to survivability, days to sprouting, sprouting percentage, leaf number, leaf length, and plant height between control and the treated plants. Moreover, the effect of different levels of gamma rays on saffron growth is illustrated in figure 4.3.

4.3.1.1 Survival rate

The results depicted in figure 4.4(A) show that the application of gamma rays resulted in a significant reduction in most of the vegetative parameters of gamma-irradiated corms as compared to non-irradiated corms. It is evident from the data that higher doses of gamma irradiation significantly ($p \leq 0.05$) reduced the survival rate in saffron corms. The maximum percentage of corm survivability (100%) reflected in control and low doses (10 Gy and 20 Gy), while 30 Gy and 40 Gy recorded minimum survival rate of 75% and 31%, respectively.

4.3.1.2 Sprouting rate

The corm sprouting sensitivity test revealed that exposure at doses higher than 20 Gy significantly reduced the percentage sprouting response of saffron corms. The highest sprouting percentage (100%) was observed in control and lower doses of gamma irradiation. The percent sprouting response decreased with increased doses of gamma rays by 19% at 30 Gy and 69% at 40 Gy as shown in figure 4.4(B).

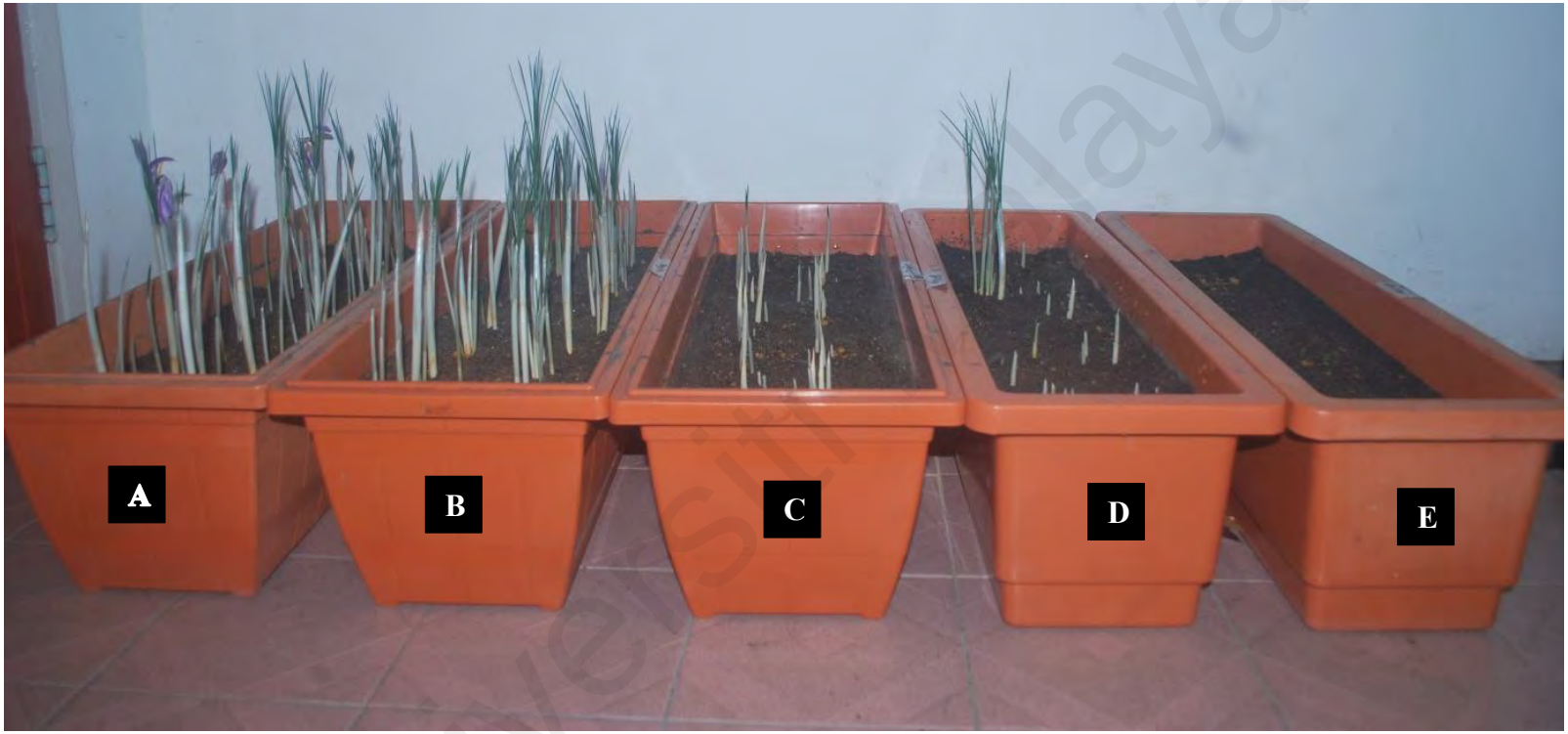


Figure 4.3: The effect of different levels of gamma rays on saffron growth, (A) 0 Gy; (B) 10 Gy; (C) 20 Gy; (D) 30 Gy and (E) 40 Gy

4.3.1.3 Days to sprouting

Gamma radiation significantly reduced and delayed sprouting response in saffron irradiated corms as shown in figure 4.5(A). Untreated control corms sprouted earlier compared to irradiated corms. There was no significant difference ($p \leq 0.05$) in days to sprouting between control (43.37 days) and 10 Gy plants (46.87 days). Corms treated with 20 Gy delayed sprouting response to 59.50 days followed by 30 Gy (79.15 days). A dose of 40 Gy induced the longest number of days to sprouting (101.20 days).

4.3.1.4 Number of leaves

Gamma irradiation of saffron affected the average number of leaves produced per plant that decreased significantly ($p \leq 0.05$) at doses higher than 10 Gy as depicted in figure 4.5(B). The highest average number of leaves (5.93) was recorded in plants treated with 10 Gy followed by untreated plants (5.18) and 20 Gy (4.68). Higher doses adversely affected leaves formation and produced 3.88 and 2.80 leaves per plant at 30 Gy and 40 Gy, respectively. Also, 40 Gy plants did not show the expected trend of saffron growth by producing true leaves directly from the shoot and produced cataphylls.

4.3.1.5 Leaf length

Irradiation of 20 Gy, 30 Gy, and 40 Gy significantly ($p \leq 0.05$) reduced leaf length as compared to the untreated and 10 Gy plants as illustrated in figure 4.6(A). The average leaf length in response to 10 Gy was highest at 14.78 cm, while the minimum average leaf length per plant (0.94 cm) was reported at 40 Gy. Untreated plants showed an average leaf length of 13.23 cm. No significant difference ($p > 0.05$) was observed in mean leaf length per plant between 10 Gy and control plants.

4.3.1.6 Plant height

Plant height reduced significantly ($p \leq 0.05$) with an increase in radiation dose as illustrated in figure 4.6(B). The growth in average plant height in response to 10 Gy was significantly highest (25.06 cm) compared to the lowest average plant height induced by 40 Gy (2.43 cm). There was no significant difference in average plant height between 10 Gy and untreated plants. Furthermore, exposure of corms at 20 Gy and 30 Gy showed an average plant height of 13.74 and 7.69 cm, respectively as compared to the control plants (26.43 cm).

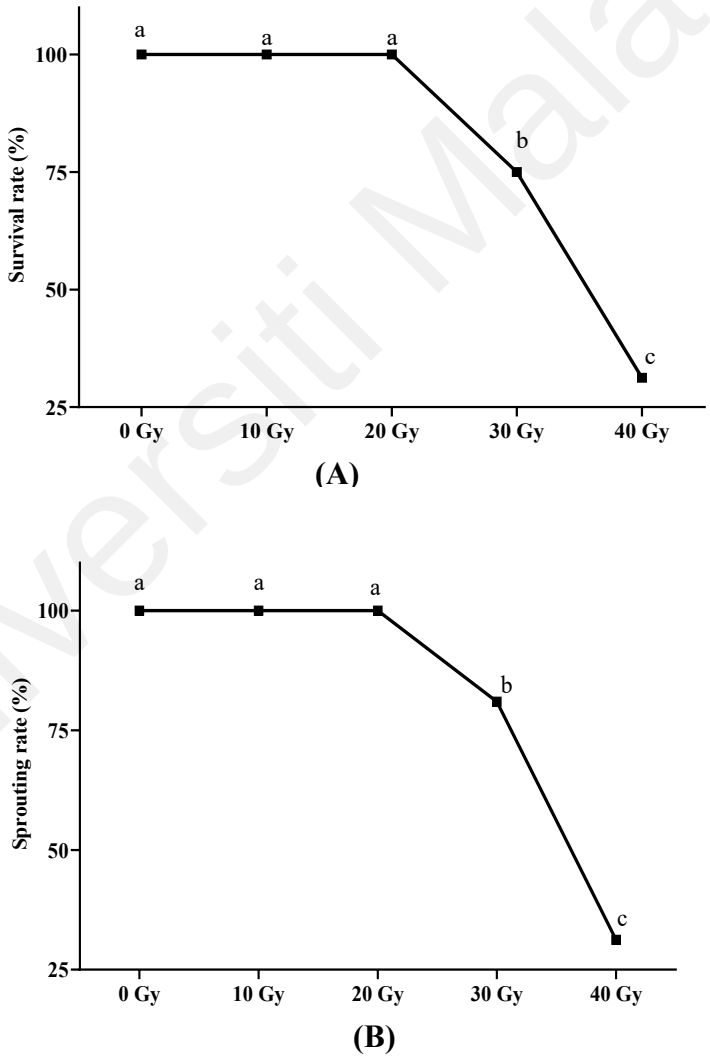


Figure 4.4: The effect of gamma irradiation on (A) survival rate; (B) sprouting rate. Means followed by different letters differ from each other at $p \leq 0.05$

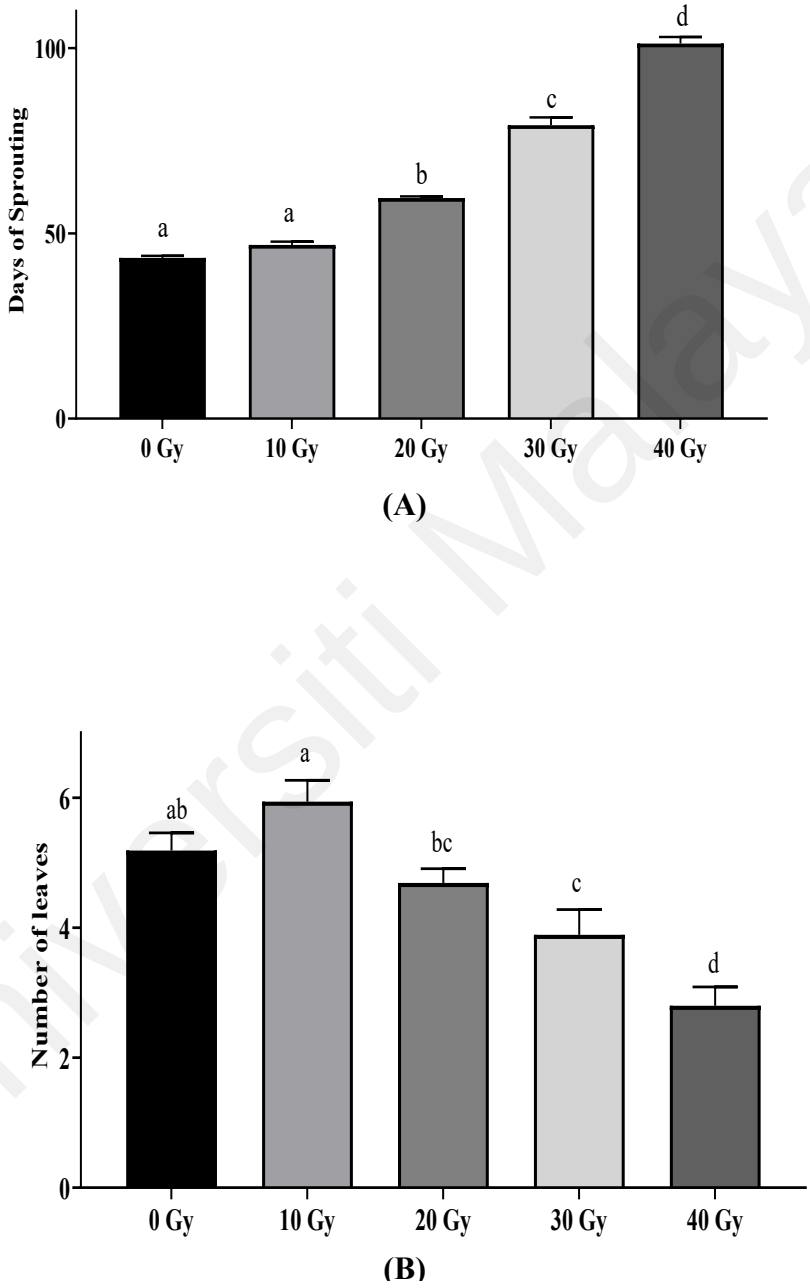


Figure 4.5: The effect of gamma irradiation on (A) days to sprouting; (B) leaf number. Vertical bars represent ± SE of the mean. Means followed by different letters differ from each other at p≤0.05

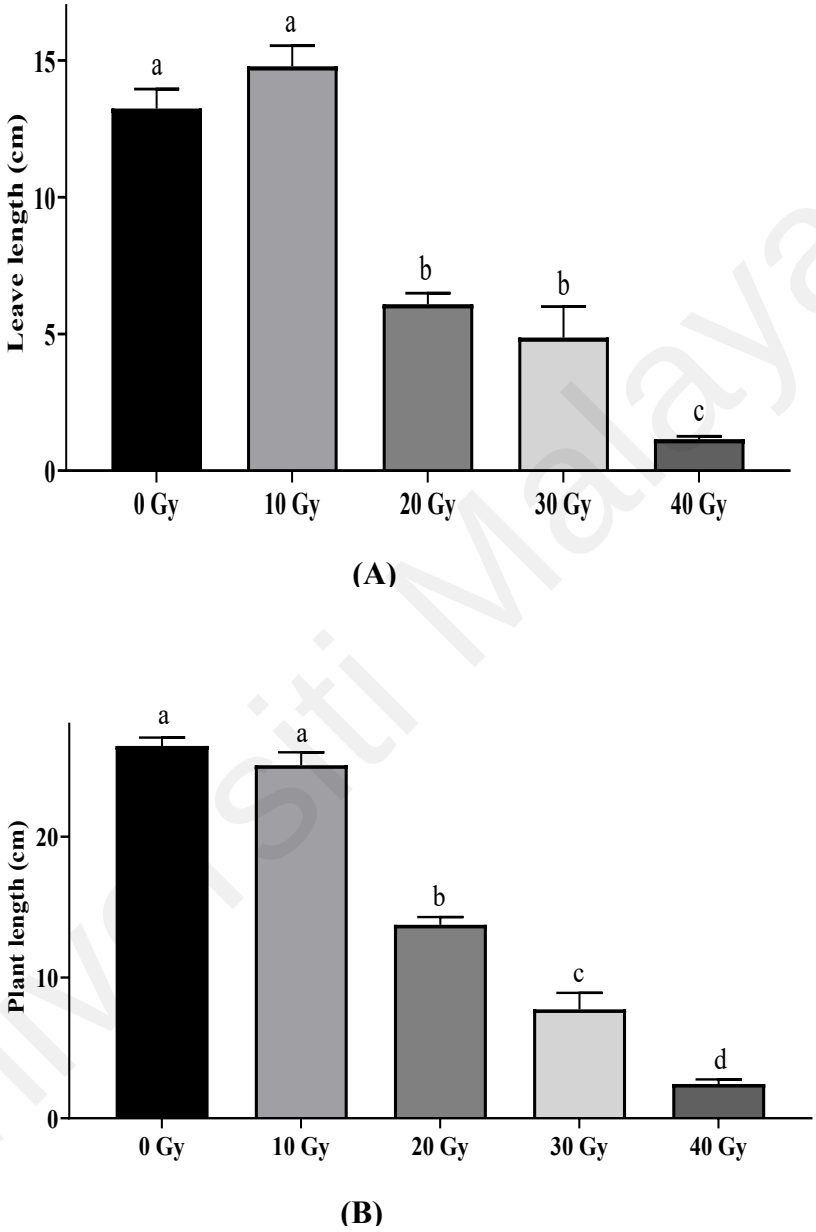


Figure 4.6: The effect of gamma irradiation on (A) leaf length; (B) plant height. Vertical bars represent ± SE of the mean. Means followed by different letters differ from each other at $p \leq 0.05$

4.3.2 Gamma radiation effect on floral parameters

The data of floral parameters of saffron interpolated in Table 4.17 shows significant differences with respect to the number of days to flower emergence, flowering duration, flower number, flower bloomed, dry flower weight, fresh flower weight, dry stigma weight, fresh stigma weight, and stigma length between control and the treated plantlets.

4.3.2.1 Days to flower emergence

As evident from Table 4.17, increasing the radiation dose significantly ($p \leq 0.05$) increased the number of days to flower emergence. Plants exposed to 10 Gy started flowering 3 days earlier (85 days) than control plants (88 days). However, no significant difference ($p > 0.05$) between 10 Gy and control plants was observed. On the contrary, irradiation of 20 Gy and 30 Gy significantly delayed the flower emergence by 101 and 107 days, respectively, while 40 Gy plants did not go into the flowering phase. Floral emergence occurred on the apical meristem of the plant.

4.3.2.2 Flower duration

Gamma irradiation affected the harvesting period in saffron statistically as shown in Table 4.17. The 10 Gy plants started flowering 4 days earlier (3rd November 2018) than control plants (7th November) and ended on 22nd November 2018, by delaying the mean harvest period from 7.9 days (control) to 8.8 days. The 20 Gy plants though started flowering on 19th November but delayed the harvest period of saffron to 8.1 days until the first week of December (2nd December 2018). On the other hand, 30 Gy plants shortened the harvest period to only 4.2 days by showing the first and last flower on 2nd December and 7th December, respectively.

Table 4.17: Effect of gamma rays on days to flowering, flowers formed, flower duration, flowers bloomed, flower fresh weight, flower dry weight, stigma fresh weight, stigma dry weight, and stigma length

Observations	Doses of gamma rays				
	0 Gy	10 Gy	20 Gy	30 Gy	40 Gy
Days to flowering (days)	88.92^{a}	85.81^{a}	101.08^{b}	107.25^{c}	0^{d}
Number of flowers formed	1.25^{a}	0.87^{b}	0.43^{c}	0.18^{cd}	0^{d}
Flower duration (days)	7.93^{a}	8.82^{a}	8.08^{a}	4.25^{b}	0^{c}
Flower blooming rate (%)	87^{a}	43^{b}	31^{b}	6^{c}	0^{c}
Flower fresh weight (mg)	150.47^{ab}	128.91^{bc}	172.90^{a}	108.87^{c}	0^{d}
Flower dry weight (mg)	32.29^{ab}	26.64^{bc}	36.37^{a}	20.78^{c}	0^{d}
Stigma fresh weight (mg)	27.25^{b}	21.32^{b}	38.84^{a}	19.17^{b}	0^{c}
Stigma dry weight (mg)	5.57^{b}	4.30^{b}	7.72^{a}	4.13^{b}	0^{c}
Stigma length (cm)	4.50^{ab}	3.97^{bc}	4.96^{a}	3.70^{c}	0^{d}

Mean values with different letters within the same column differ significantly from each other at $p \leq 0.05$.

4.3.2.3 Number of flowers formed

Results obtained for the number of flowers per planted corm indicated that gamma rays inhibited flower formation significantly (Table 4.17). Among the gamma-irradiated corms, the best results comparable with the control (1.25) were obtained for 10 Gy, showing maximum number of flowers per corm (0.87) associated with the corresponding decline at higher doses. Irradiation with 20 Gy and 30 Gy recorded mean flower number of 0.43 and 0.18, respectively.

4.3.2.4 Flower blooming rate

The stage of full bloom begins when 80% of flower mass is opened as shown in Fig. 4.7(B). As shown, gamma irradiation negatively affected flower blooming (Table 4.17). The highest blooming percentage (87%) was obtained in untreated plants. Plants treated with 10 Gy recorded a flower blooming rate of 43%, while 20 Gy and 30 Gy exhibited blooming percentages of 31% and 6%, respectively.

4.3.2.5 Flower fresh weight

The fresh flower weight per plant was significantly affected by various doses of gamma irradiation as shown in Table 4.17. The highest flower fresh weight of 172.90 mg was obtained in plants developed with 20 Gy exposure followed by untreated plants (150.47 mg). There was no significant difference ($p>0.05$) in fresh flower weight between 20 Gy and untreated controlled plants. Irradiations with 10 Gy and 30 Gy reduced the fresh flower weight from 128.91 to 108.87 mg, respectively.

4.3.2.6 Flower dry weight

Likewise, flower dry weight per plant followed a similar pattern of response to gamma irradiation. Flower dry weight increased when the total dose was increased from 10 Gy to 20 Gy showing a maximum flower dry weight of 36.37 mg at 20 Gy. However, a similar trend was not observed when the dose was increased beyond 20 Gy. Table 4.17 illustrates that a very low dose (10 Gy) and high dose (30 Gy) negatively affected flower dry weight with values of 26.64 mg and 20.78 mg, respectively compared with that of control (32.29 mg).

4.3.2.7 Stigma fresh weight

Unlike other floral characters, stigma fresh weight in response to 20 Gy was significantly highest at 38.84 mg per flower compared to the control plant of 27.25 mg (Fig. 4.8). On the other side, 10 Gy followed by higher doses of 30 Gy and 40 Gy revealed an inhibitory effect on stigma fresh weight (Table 4.17). Among all the flower-bearing plants produced, 30 Gy showed the least stigma fresh weight of 19.17 mg per flower followed by 10 Gy (21.32 mg).

Figure 4.7: Stages of flower formation (A) flower emerged; (B) flower bloomed

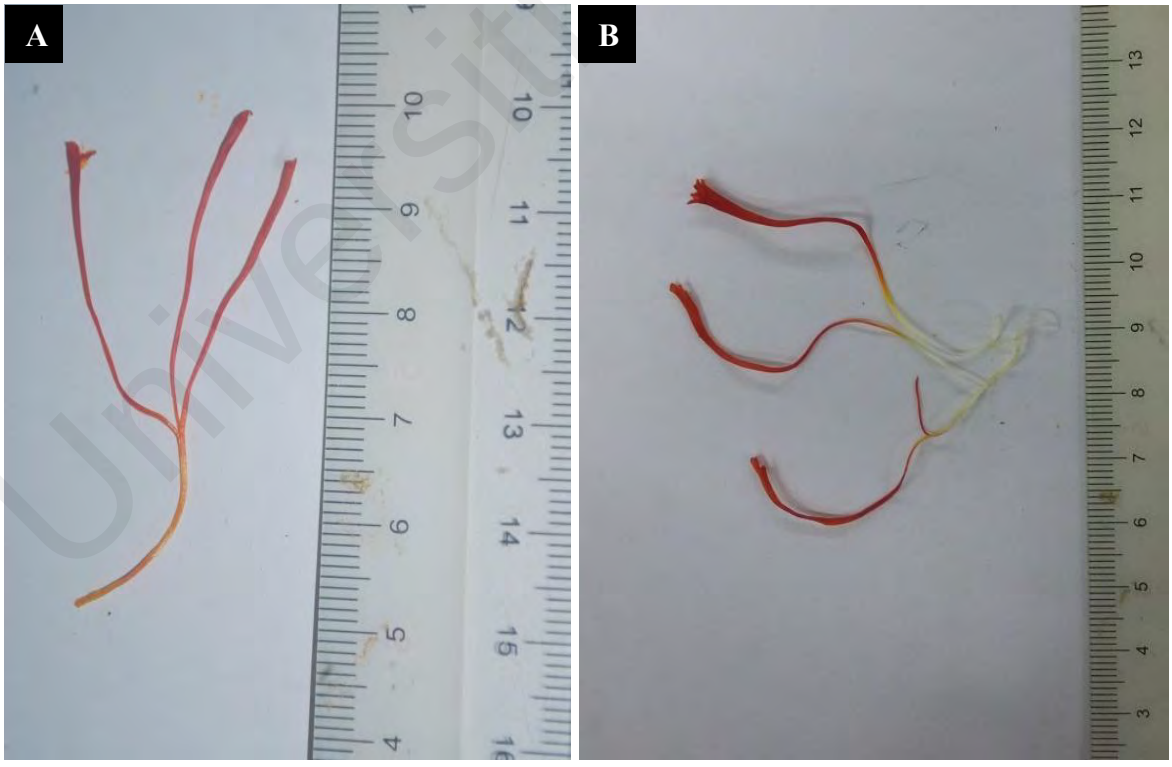


Figure 4.8: Three stigmatic lobes of saffron (A) Control; (B) 20 Gy

4.3.2.8 Stigma dry weight

It was observed that exposure of 20 Gy stimulated a significant increase in dry stigma weight of saffron per corm as compared to other doses and control plants. The maximum amount of dry stigma weight (7.72 mg) per flower was obtained at 20 Gy. As expected, irradiation dose exceeding 20 Gy negatively affected floral characters. Exposure of corms at 10 Gy and 30 Gy attenuated stigma dry weight from 4.30 mg to 4.13 mg per flower, showing no significant difference with the control (5.57 mg).

4.3.2.9 Stigma length

The length of stigma with respect to different doses of gamma radiation is given in Table 4.17. The saffron plants differed significantly in response to stigma length among the various levels of gamma rays (Fig. 4.8). The 20 Gy resulted in the maximum stigma length of 4.96 cm. On the contrary, 10 Gy and 30 Gy plants reduced the length of stigma and showed an average stigma length of 3.97 cm and 3.70 cm, respectively compared to the untreated plants of 4.50 cm.

4.3.2.10 Floral shape variation

Flower shape variation in irradiated and control plants was also observed. The appraisal of Fig. 4.9 shows that gamma irradiation of saffron corms extensively affected floral organs. In the present study, 20 Gy dose was found good in the induction of useful mutation for flower color and shape showing four petals, irregularly shaped petals, tetrafid stigma, and two stamens in one flower (Fig. 4.9B) compared with six normal-sized petals, trifid stigma, and three stamens in control as shown in Fig. 4.9(A). Also, most of the petals of 20 Gy mutated plant were darker in color compared to the control plants.

Figure 4.9: Flower of C. sativus (A) Normal flower; (B) Flower variation after 20 Gy

4.3.3 Gamma radiation effect on corm growth parameters

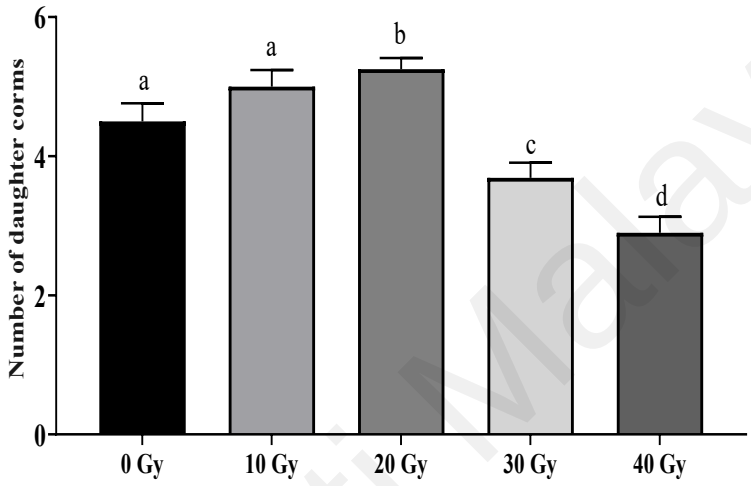
4.3.3.1 Number of progeny corms

It is obvious from the data in figure 4.10(A) that the total number of progeny corms increased with increasing doses of gamma irradiation up to 20 Gy and recorded maximum progeny corms at 20 Gy (5.25) followed by 10 Gy (5.00). On the other hand, higher irradiation doses significantly decreased the number of progeny corms, and the lowest number of corms was obtained at 40 Gy (2.70) and 30 Gy (3.85) as compared with the control plants (4.50).

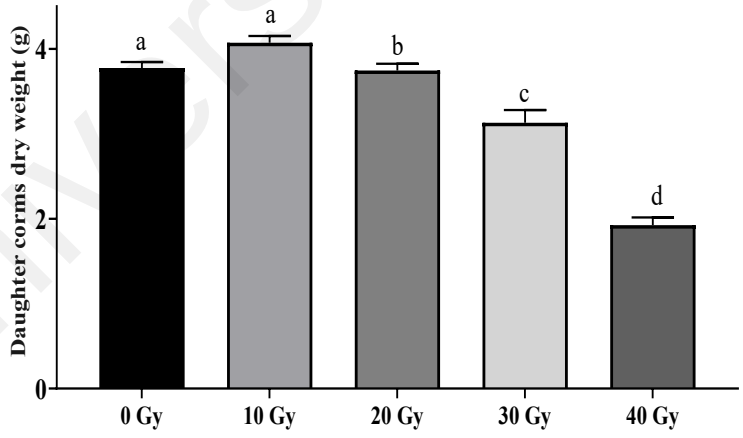
4.3.3.2 Weight of progeny corms

The weight of progeny corms with respect to different levels of gamma rays is shown in Fig. 4.10(B). The results revealed that the weight of daughter corms decreased significantly

with increasing doses of gamma rays. Overall, plants induced by 10 Gy exhibited the highest significant weight of progeny corms (4.07 g) compared to the lowest weight of progeny corms at 40 Gy (2.02 g). Likewise, 20 Gy and 30 Gy produced daughter corms weighing 3.74 and 3.34, respectively. However, there was no significant difference in progeny corms weight of control plants and 10 Gy to 30 Gy plants.



(A)



(B)

Figure 4.10: The effect of gamma irradiation on saffron corm growth attributes (A) number of daughter corms; (B) weight of daughter corms produced. Vertical bars represent ± SE of the mean. Means followed by different letters differ from each other at p≤0.05

4.4 Oral acute toxicity assay

4.4.1 Cage side observation

As evident from table 4.18, none of the experimental groups of mice showed any abnormal behavioral, motor, and neuronal functions for all the administered ethanolic extracts. Also, no mortality or abnormality was observed. The monitoring of eyes, skin and fur, behavioral pattern, and autonomic and central nervous system activities of treated mice remained unchanged with the treatment of saffron extracts when compared with the normal control group. No apparent morphological alterations were observed in saffron treated mice. Hence, LD_{50} for oral route of all saffron ethanolic extracts was estimated to be greater than 2 g/kg b.w.

4.4.2 Body weight measurement

Weekly examined body weights of mice administered with saffron ethanolic extracts were comparable to the normal control group in both genders. Body weight fluctuation was observed throughout the experiment, but statistically no significant ($p>0.05$) change in body weight was recorded when compared controlled group with saffron treated groups showing that 2 g/kg saffron treatment has no major effect on body weight as illustrated in table 4.19.

4.4.3 Water and feed intake

The water and feed intake were observed to be regular and consistent across all treatment groups throughout the experimental period in both sexes, as compared to the normal control group as illustrated in table 4.20 and 4.21. This indicates that saffron ethanolic extract has no major effect on water and food intake.

4.4.4 Hematological analysis

The data of a single oral administration of 2000 mg/kg b.w dose of CEE, LEE, PEE and SEE on hematological parameters of mice is shown in table 4.22. The hematological parameters of the male and female mice revealed significant ($p<0.05$) increase in platelet count ($616.6x10^3/\mu L$) of SEE treated male group compared to the control group ($548.4x10^3/\mu L$). However, these differences were not considered to be toxicologically meaningful. No significant difference in other hematological parameters of animals were observed.

4.4.5 Biochemical analysis

The changes in biochemical parameters for both sexes with oral administration of 2000 mg/kg body weight dose of CEE, LEE, SEE and PEE compared to their respective control group are given in figure 4.11, 4.12 and 4.13. Biochemical analysis showed no significant difference ($p>0.05$) in any of the parameters analyzed in either control or treatment group of both female and male mice. Nonetheless, the amounts of all biochemical parameters were a bit higher in PEE treated female mice as compared with the controlled group. The PEE treated male mice showed same pattern except ALT and TC that were lowered compared to the control group. Yet, this difference is not significant statistically ($p>0.05$).

4.4.6 Relative organ weight

The ROW of dissected organs (liver, kidneys, pancreas, and heart) of treated and untreated control mice of either sex is given in Fig 4.14 and 4.15. The differences in ROW recorded between the treatment and the control groups were not significant statistically. However, the ROW of liver, pancreas, and heart in SEE treated mice of either sex was slightly higher than untreated group. On the contrary, the ROW of liver, kidneys and heart in PEE treated mice

of both sexes was slightly lower than the control. Yet, this difference is not statistically ($p>0.05$) significant.

4.4.7 Histopathological analysis

Histopathological features of heart, pancreas and kidney sections of treated and untreated group are shown in figure 4.16 and 4.17. Light microscopic examination of tissues from both sexes of saffron treated mice showed a normal histology without any adverse effect on histological attributes of the vital organs and was similar to that of the control group mice. The photomicrographs of the heart sections in saffron treated mice revealed no abnormalities in the cardiomyocyte architecture. The normal architecture of the cardiac myocytes was maintained with the absence of cardiac myopathy, necrosis, myofiber degeneration, vacuolation and mononuclear cell infiltration showing that saffron administration had no toxic effect on the heart of both male and female mice. Similarly, all kidney sections displayed normal glomerular and unremarkable typical tubule interstitial parenchyma, with no hyaline changes or vascular necrosis.

Table 4.18: Behavioral responses and general appearance of mice treated with saffron methanolic extracts in acute toxicity study

Observation	Control	CEE	LEE	PEE	SEE
Male					
Temperature	Normal	Normal	Normal	Normal	Normal
Urination	Normal	Normal	Normal	Normal	Normal
Skin colour	No effect	No effect	No effect	No effect	No effect
General physique	Normal	Normal	Normal	Normal	Normal
Drowsiness	Not present	Not present	Not present	Not present	Not present
Movement	Normal	Normal	Normal	Normal	Normal
Hyperactivity	Normal	Normal	Normal	Normal	High
Mortality	Not found	Not found	Not found	Not found	Not found
Female					
Temperature	Normal	Normal	Normal	Normal	Normal
Urination	Normal	Normal	Normal	Normal	Normal
Skin colour	No effect	No effect	No effect	No effect	No effect
General physique	Normal	Normal	Normal	Normal	Normal
Drowsiness	Not present	Not present	Not present	Not present	Not present
Movement	Normal	Normal	Normal	Normal	Normal
Hyperactivity	Normal	Normal	Normal	Normal	High
Mortality	Not found	Not found	Not found	Not found	Not found

Table 4.19: Effect of saffron ethanolic extracts on body weight (g) of mice during 14 days treatment

Sex	Days	Control	CEE	LEE	PEE	SEE
Male	1st	26.55±2.10	25.97±2.30	26.31±1.10	25.57±0.80	26.40±0.90
	7th	28.81±1.30	27.98±1.60	29.06±1.30	28.12±1.50	29.01±2.10
	14th	29.63±2.20	29.06±2.10	30.72±3.20	29.84±1.90	30.57±2.90
Female	1st	25.63±1.80	26.02±1.90	25.90±1.70	24.97±1.20	25.42±1.50
	7th	26.86±2.10	27.09±1.80	27.29±1.80	26.35±1.70	26.94±1.70
	14th	28.24±2.40	28.47±2.20	29.03±2.30	27.68±2.20	28.43±2.10

Values are presented as Mean ± SD (n=5). $p>0.05$ (One-way ANOVA)

Table 4.20: Effect of saffron ethanolic extracts on food intake (g) of mice during 14 days treatment

Sex	Days	Control	CEE	LEE	PEE	SEE
Male	1st	8.49±0.83	7.10±1.10	7.92±1.40	9.08±2.50	8.36±2.20
	7th	7.82±0.87	7.68±1.30	8.04±2.10	8.65±2.30	8.59±2.30
	14th	8.15±1.20	7.26±1.20	7.84±0.98	8.58±2.40	8.66±2.60
Female	1st	7.85±0.91	8.07±2.10	7.09±0.83	8.35±1.90	8.96±2.40
	7th	8.54±0.94	8.34±2.00	7.27±0.87	8.48±2.10	8.72±2.50
	14th	8.44±1.30	8.22±0.99	6.89±0.82	7.92±1.80	8.18±2.30

Values are presented as Mean ± SD (n=5). p>0.05 (One-way ANOVA).

Table 4.21: Effect of saffron ethanolic extracts on water intake (ml) of mice during 14 days treatment

Sex	Days	Control	CEE	LEE	PEE	SEE
Male	1st	8.1±1.60	7.8±1.90	7.5±1.50	8.0±2.20	11.6±2.40
	7th	7.6±1.10	7.4±1.70	7.8±1.20	6.8±1.30	9.1±0.99
	14th	8.2±2.10	7.2±1.60	8.0±1.50	7.7±1.70	8.5±1.50
Female	1st	8.5±2.00	7.7±1.80	8.0±1.50	7.9±1.20	10.8±2.20
	7th	8.2±2.30	7.9±2.10	7.5±1.30	8.2±1.00	9.4±0.92
	14th	7.9±1.90	8.2±2.20	7.6±1.10	8.4±2.90	8.1±0.83

Values are presented as Mean ± SD (n=5). p>0.05 (One-way ANOVA).

Table 4.22: Hematological parameters of mice orally treated with saffron ethanolic extracts for 14 days

Parameters	Control	CEE	LEE	PEE	SEE
Male					
RBC ($x10^6$/μL)	9.09±0.61	9.20±0.56	8.92±0.63	9.56±0.64	10.03±0.73
Hb (g/dL)	12.93±0.43	13.95±0.51	12.91±0.62	13.98±0.48	14.07±0.82
HCT (%)	41.98±1.20	42.84±2.30	39.50±2.20	43.86±2.70	44.22±3.20
MCV (fL)	52.80±2.30	53.36±3.60	50.58±2.10	53.9±2.80	55.32±3.30
MCH (ρg)	15.18±1.40	15.26±0.53	14.56±1.20	15.08±0.84	15.34±0.93
MCHC (g/dL)	31.46±1.10	31.62±0.65	29.94±1.80	31.42±2.20	31.50±0.26
RDW-CV (%)	16.06±1.50	17.4±1.80	17.28±0.92	16.84±1.10	17.72±0.59
RDW-SD (fL)	25.92±1.80	26.82±2.10	27.48±1.30	26.48±1.90	28.66±1.80
Plt ($x10^3$/μL)	548.40±2.46	571.20±3.53	543.80±3.62	584.60±3.09	616.60±4.06*
WBC ($x10^3$/μL)	6.02±0.72	5.64±0.41	6.39±0.57	6.17±1.60	6.43±0.61
Neu (%)	15.72±2.10	18.84±2.20	15.64±1.40	16.40±1.20	14.54±2.50
Lym (%)	81.74±2.80	78.78±1.40	81.86±2.30	80.74±0.84	82.82±3.20
Mon (%)	1.54±0.23	1.28±0.22	1.54±0.32	1.70±0.40	1.42±0.35
Eos (%)	0.70±0.20	0.82±0.30	0.76±2.50	0.88±0.12	0.90±0.22
Bas (%)	0.24±0.11	0.26±0.14	0.20±0.10	0.28±0.12	0.32±0.16
Female					
RBC ($x10^6$/μL)	8.22±0.52	7.89±0.62	7.99±0.24	8.35±0.33	8.24±0.18
Hb (g/dL)	12.37±0.49	11.58±0.38	12.23±0.44	12.63±0.67	12.49±0.35
HCT (%)	39.86±1.40	37.86±0.82	36.48±1.20	41.18±1.60	40.22±1.50
MCV (fL)	49.78±1.30	47.82±1.20	47.20±1.40	51.42±0.82	50.52±1.90
MCH (ρg)	16.24±0.65	16.90±0.28	15.54±0.47	15.20±0.41	16.08±0.44
MCHC (g/dL)	32.04±1.20	32.68±0.81	31.40±0.53	30.18±0.32	31.12±0.40
RDW-CV (%)	15.82±0.63	15.76±0.54	16.52±0.47	16.06±0.38	16.44±0.82
RDW-SD (fL)	25.74±0.51	25.90±0.62	27.06±0.53	25.52±0.75	26.58±1.50
Plt ($x10^3$/μL)	497.60±4.20	461.20±3.49	473.20±2.25	522.40±2.02	509.20±2.04
WBC ($x10^3$/μL)	5.96±0.47	5.76±0.27	5.86±0.39	6.10±0.22	6.06±0.19
Neu (%)	16.90±2.60	18.66±0.53	17.98±0.86	16.32±0.55	16.26±1.40
Lym (%)	80.12±3.30	78.32±1.20	79.40±2.40	81.18±2.20	81.08±1.70
Mon (%)	1.70±0.50	1.78±0.34	1.44±0.37	1.46±0.33	1.56±0.51
Eos (%)	0.86±0.21	0.88±0.32	0.74±0.33	0.76±0.26	0.80±0.22
Bas (%)	0.40±0.10	0.36±0.11	0.36±0.14	0.28±0.02	0.30±0.10

Values are presented as Mean ± SD (n=5). $p>0.05$ (One-way ANOVA).

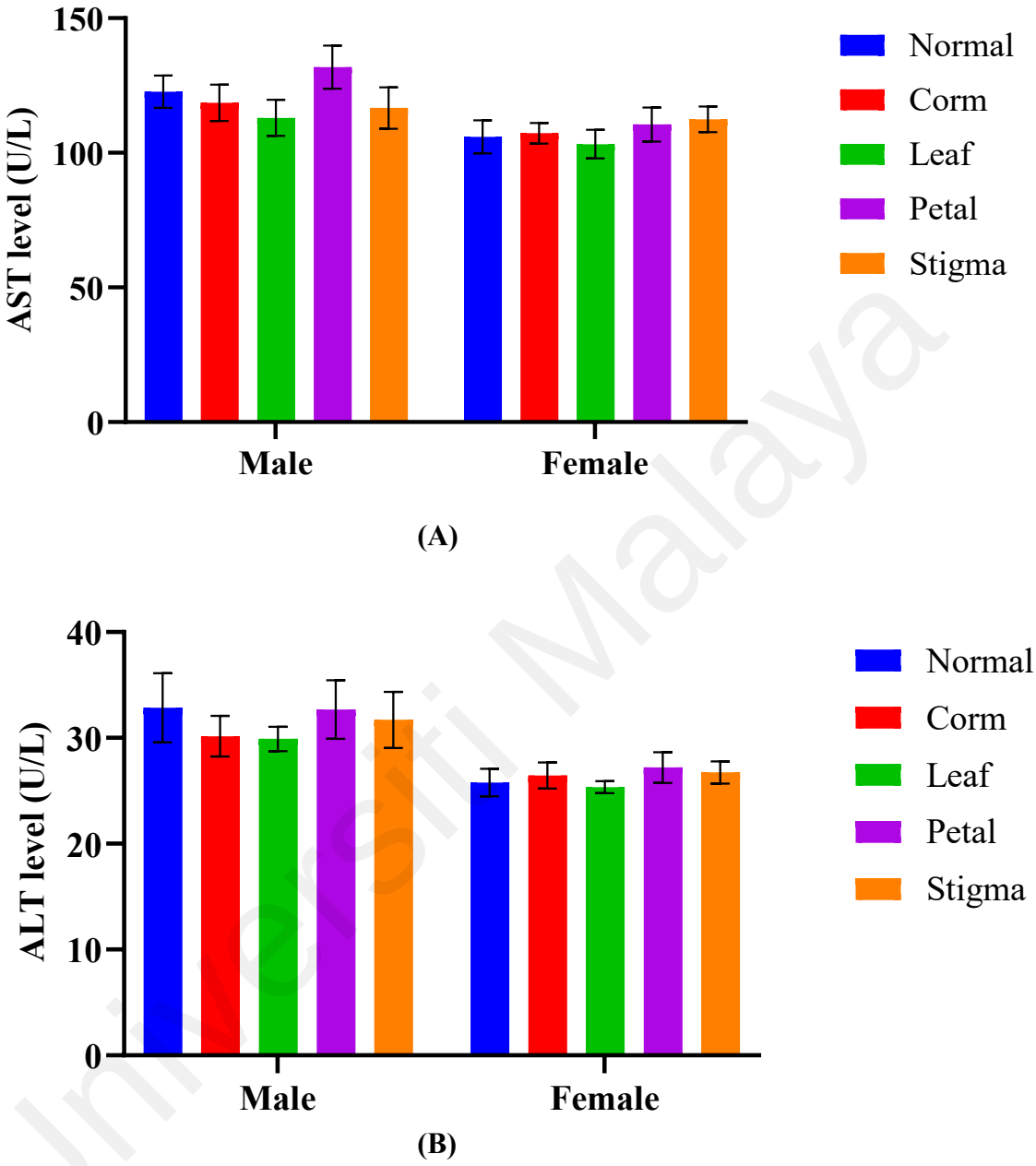


Figure 4.11: Effect of 2 g/kg saffron extracts on (A) AST and (B) ALT level in mice. Values are presented as mean ± SEM where * p<0.05 when compared with the vehicle control group

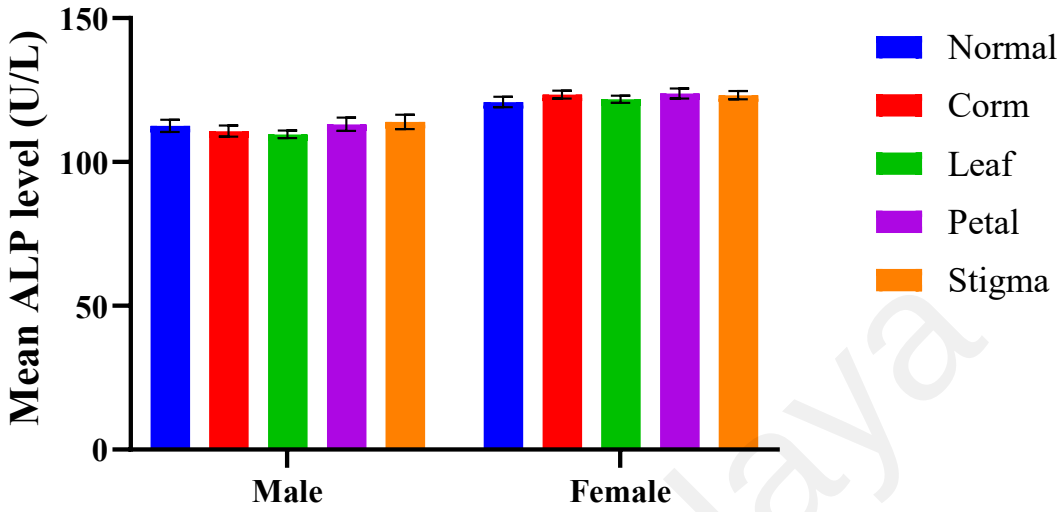


Figure 4.12: Effect of 2 g/kg saffron extracts on ALP level in mice. Values are presented as mean ± SEM where * p<0.05 when compared with the vehicle control group

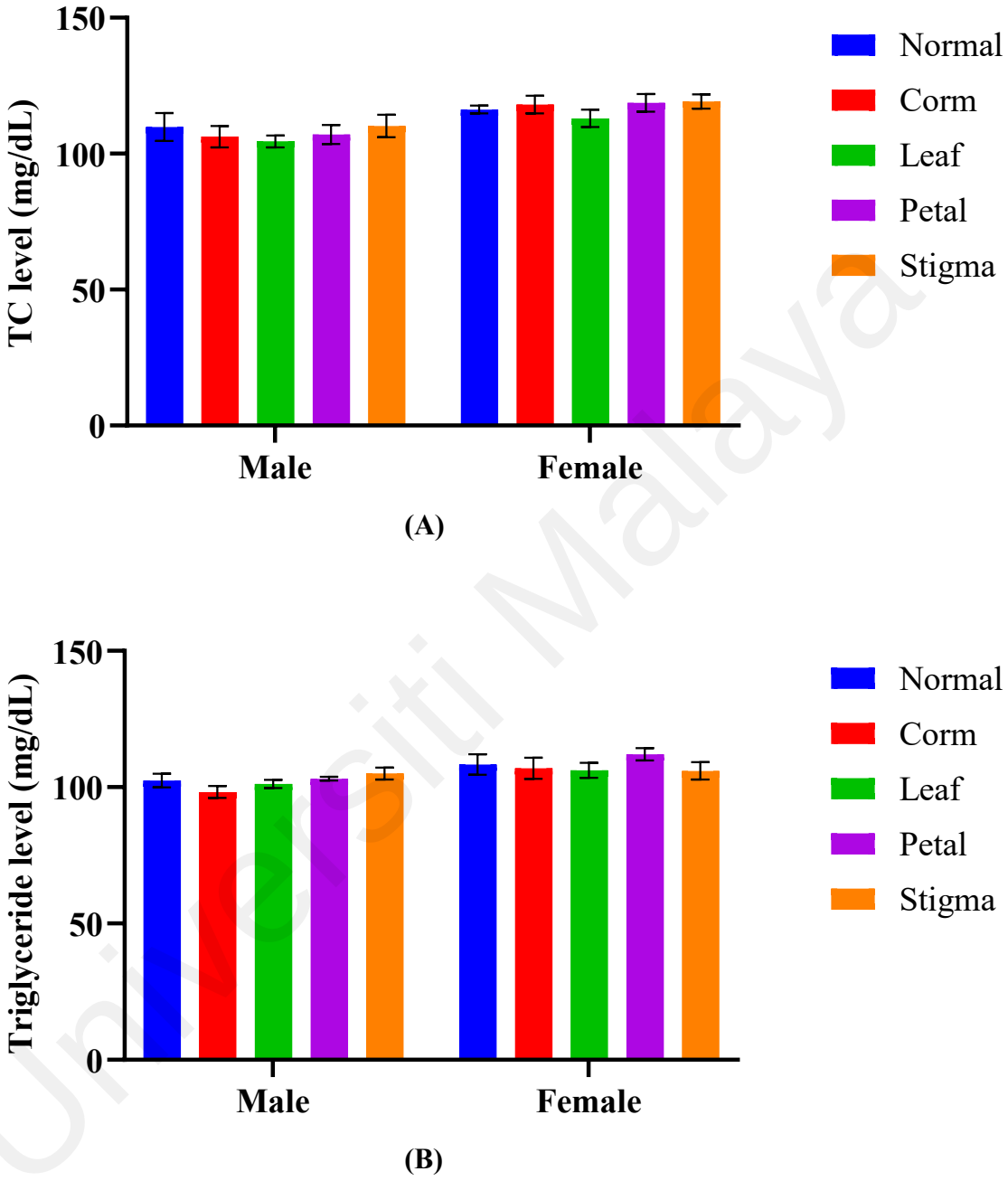


Figure 4.13: Effect of 2 g/kg saffron extracts on (A) total cholesterol and (B) triglyceride level in mice. Values are presented as mean ± SEM where * $p<0.05$ when compared with the vehicle control group

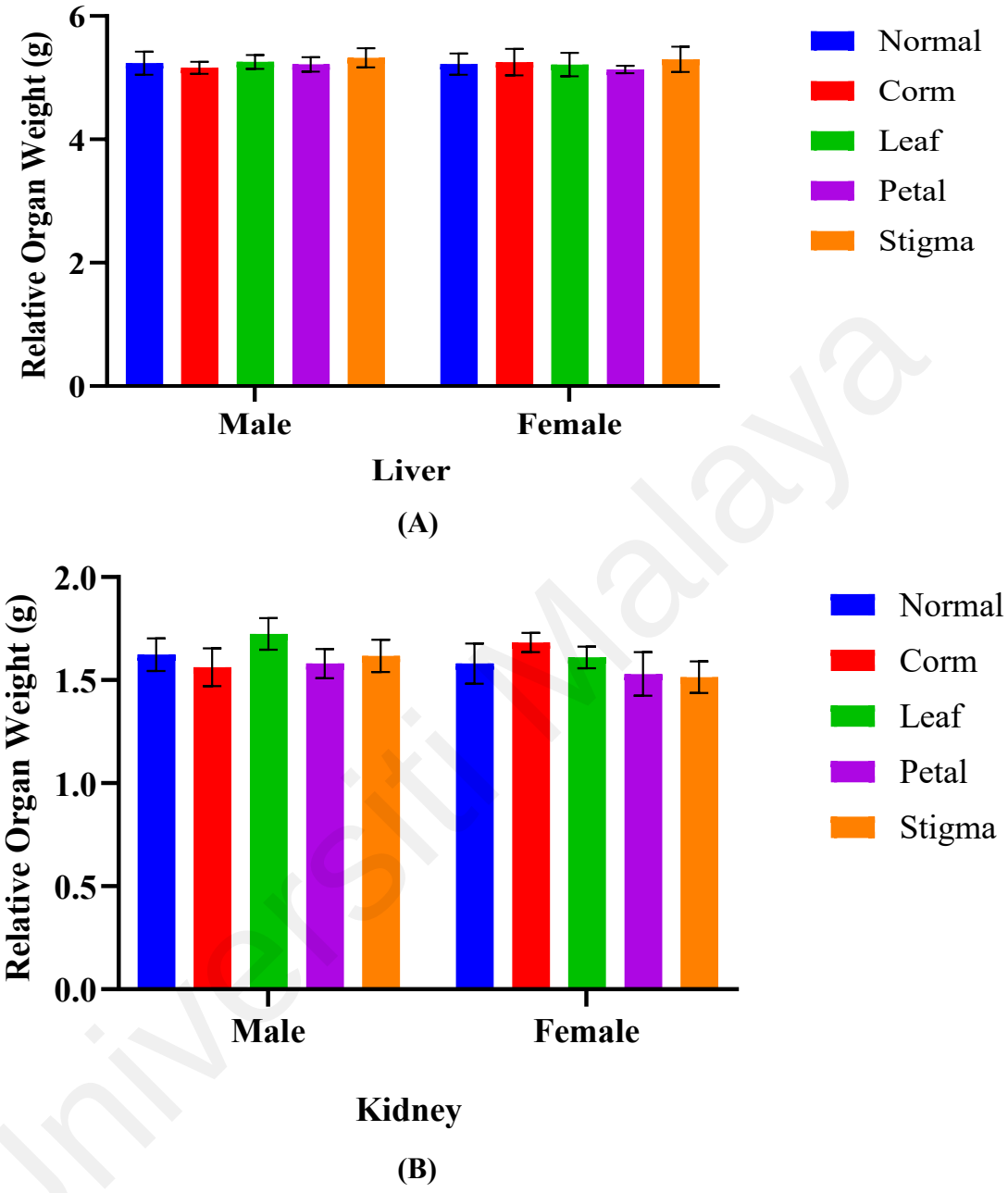


Figure 4.14: Relative organ weight of (A) liver and (B) kidney of saffron treated male and female mice after 14 days. Values are presented as mean ± SEM where * $p<0.05$ when compared with the vehicle control group

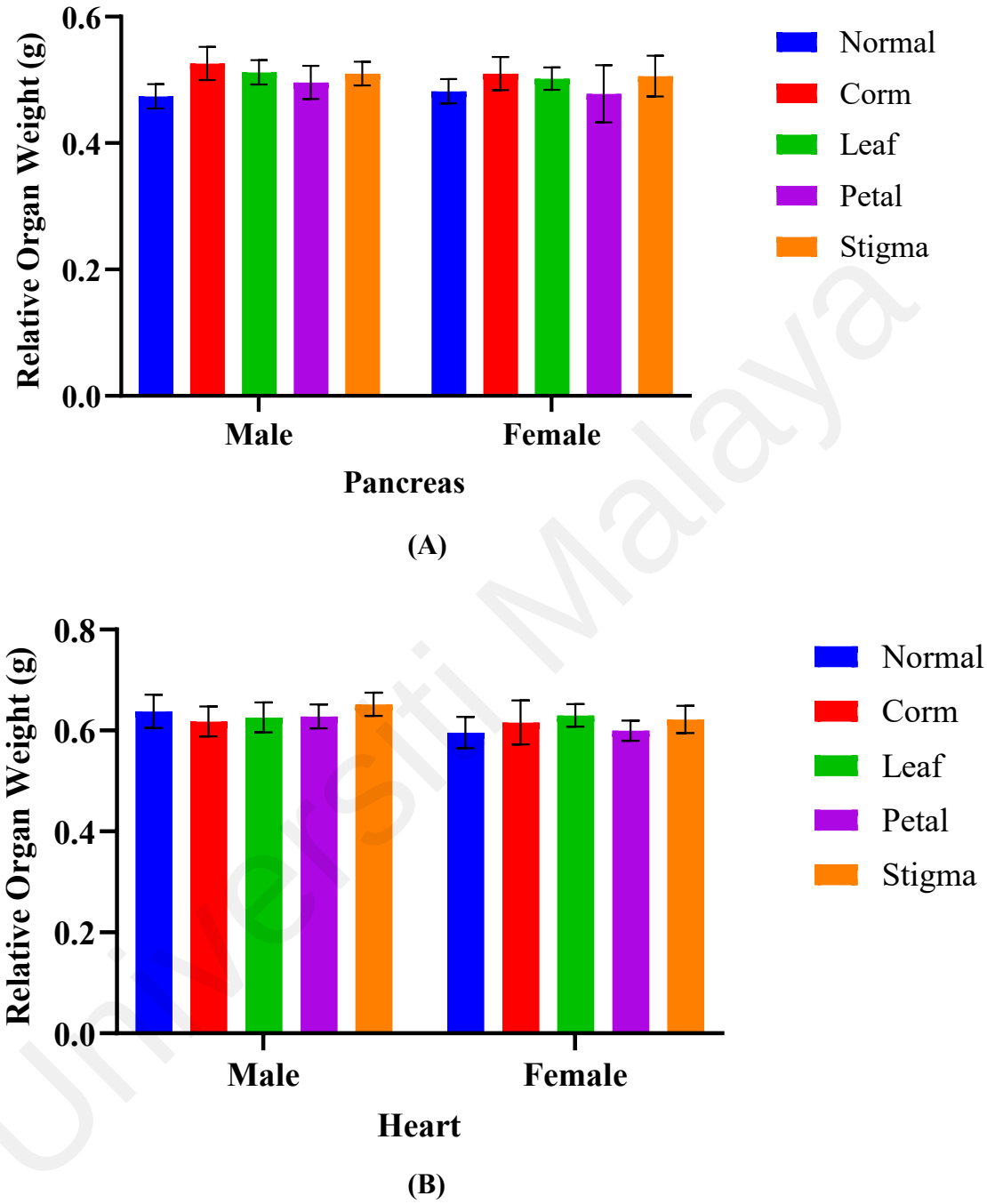


Figure 4.15: Relative organ weight of (A) pancreas and (B) heart of saffron treated male and female mice after 14 days. Values are presented as mean ± SEM where * p<0.05 when compared with the vehicle control group

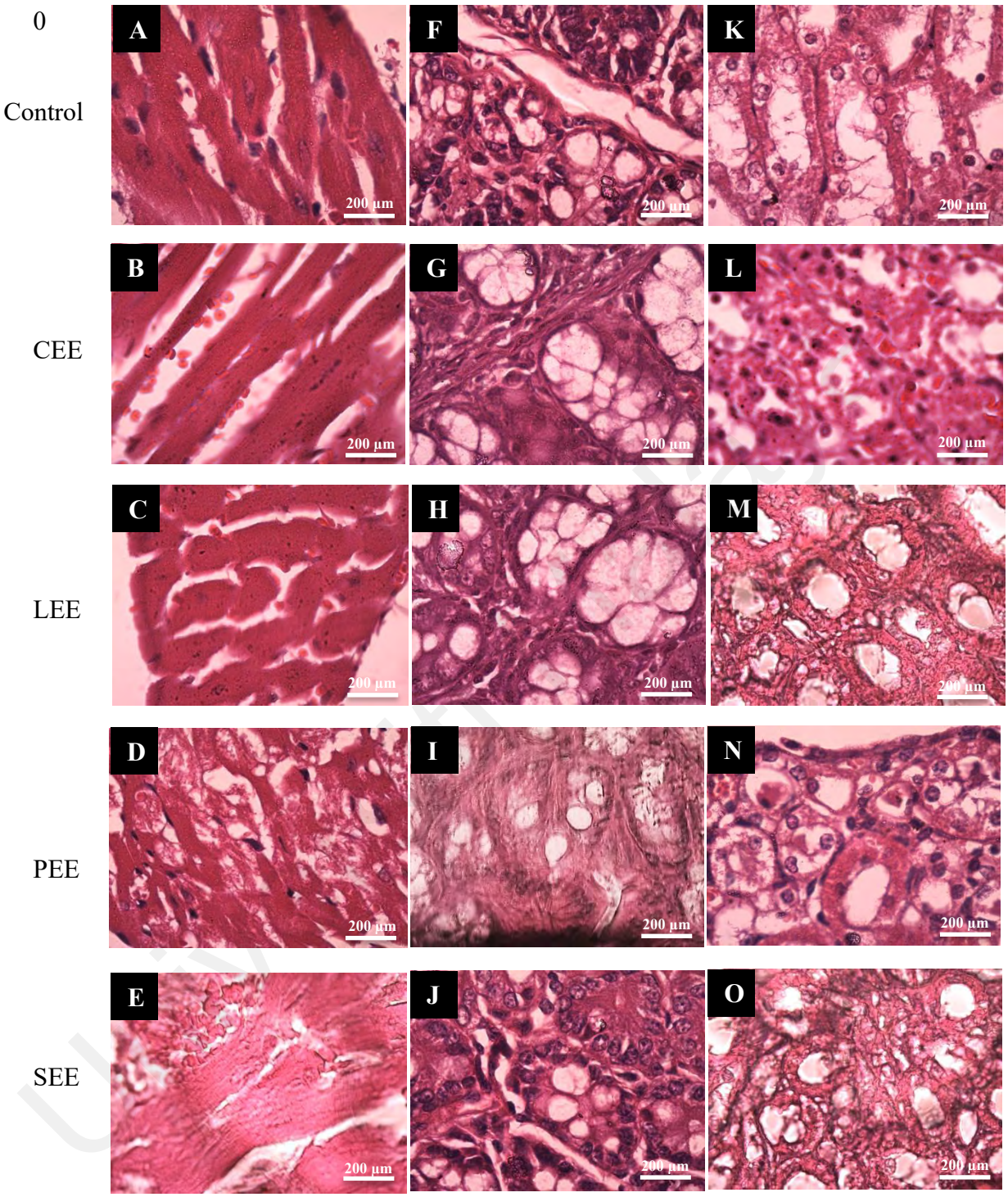


Figure 4.16: Photomicrographs of a section of heart (A-E), pancreas (F-J) and kidney (K-O) after oral administration of Control, CEE, LEE, PEE and SEE in male mice for 14 days

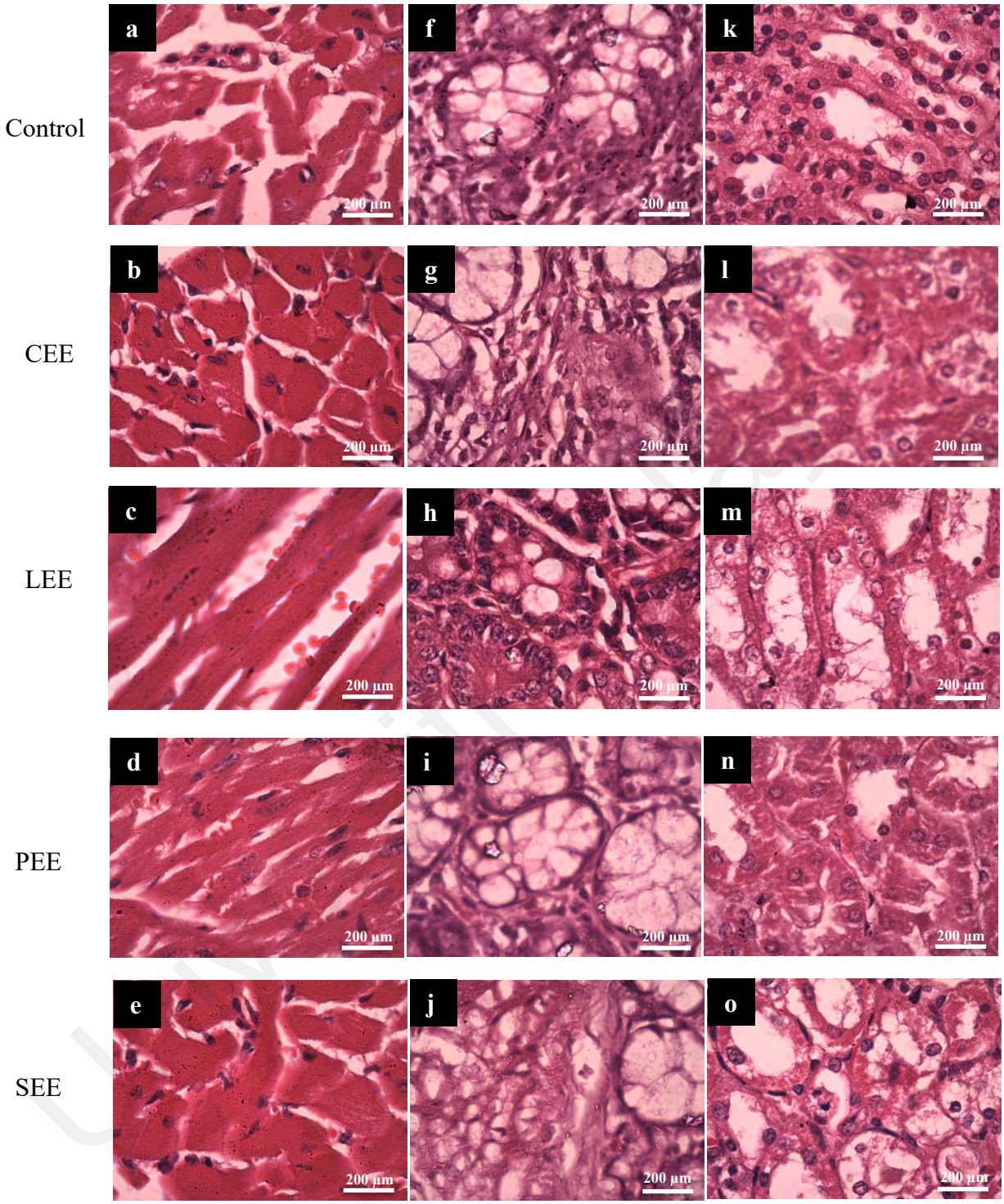


Figure 4.17: Photomicrographs of a section of heart (A-E), pancreas (F-J) and kidney (K-O) after oral administration of Control, CEE, LEE, PEE and SEE in female mice for 14 days

4.5 Pharmacological activities of saffron

4.5.1 Hot plate analgesic test

The test is based on the use of thermal stimuli to determine the effect of analgesics. For this purpose, an easy and cost-effective method, hot plate analgesic test was performed. Results of saffron ethanolic extracts presented in the form of latency time indicated that all the extracts showed significant ($P<0.05$) analgesic effect in a time-dependent manner (table 4.23). Aspirin, used as a reference drug, showed the highest latency (17.51 ± 0.50) at 1 h followed by reduction (16.82 ± 0.45) at 2 h. Among all the extracts of saffron tested, SEE showed highest latency activity (7.80 ± 0.16, 11.30 ± 0.21, 12.80 ± 0.33 and 13.50 ± 0.28) at 0, 0.5, 1 and 2 h, respectively. On the other hand, LEE exhibited no significant difference at 0 h when compared with negative control (saline) but showed the least activity of 5.92 ± 0.14, 6.22 ± 0.15, and 6.65 ± 0.19 at 0.5, 1 and 2 h, respectively. Based on the percent inhibition as shown in figure 4.18, SEE was more prominent in reducing analgesia (44.91%, 63.89%, and 72.85%) followed by PEE showing 38.84%, 53.07% and 64.06% inhibition at 0.5, 1 and 2 h, respectively. Furthermore, CEE and LEE responded poorly and showed weak analgesic activity of 22.40% and 29.07% at 2h, respectively.

Table 4.23: Latency time of *C. sativus* ethanolic extracts in hot plate assay

Group	Dose (mg/kg)	Latency time (s)			
		0 h	0.5 h	1 h	2 h
Saline	1 ml/kg	4.70 ± 0.06	5.00 ± 0.02	5.50 ± 0.01	5.40 ± 0.04
Aspirin	10	9.45 ± 0.28***	16.38 ± 0.42***	17.51 ± 0.50***	16.82 ± 0.45***
CEE	800	6.20 ± 0.17***	6.93 ± 0.16***	7.15 ± 0.13***	7.58 ± 0.14***
LEE	800	5.15 ± 0.15	5.92 ± 0.14**	6.22 ± 0.15*	6.65 ± 0.19**
SEE	800	7.80 ± 0.16***	11.30 ± 0.21***	12.80 ± 0.33***	13.50 ± 0.28***
PEE	800	6.78 ± 0.17***	9.42 ± 0.24***	10.38 ± 0.29***	11.13 ± 0.35***

Each value is represented as mean ± S.D. Where, *p<0.05, **p<0.01, ***p<0.001 statistically significant relative to control (saline)

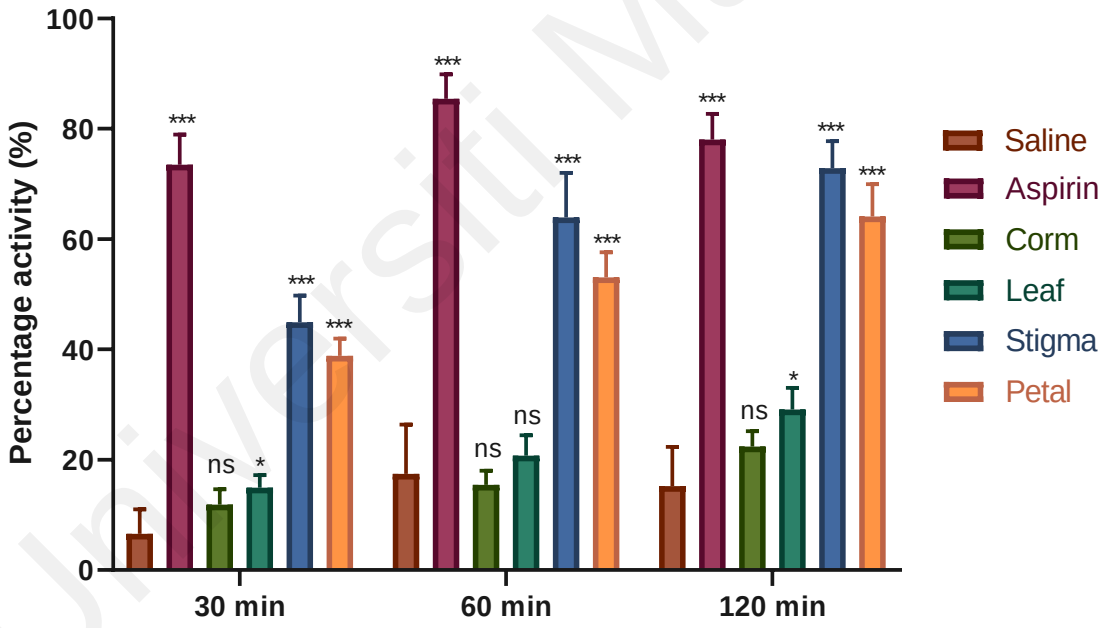


Figure 4.18: Percentage analgesia of C. *sativus* ethanolic extracts at selected time period in mice. Each value is represented as mean ± S.D. Where, *p<0.05, **p<0.01, ***p<0.001 statistically significant relative to control (saline)

4.5.2 Carrageenan-induced hind paw edema test

Anti-inflammatory effects of saffron ethanolic extracts were investigated by mouse paw-edema test and the findings are presented in table 4.24. Sub planter injection of carrageenan gradually increased edema paw volume of the saline-treated animals. However, as a positive control, diclofenac potassium attenuated paw edema volume by 88.87% as depicted in figure 4.19. Moreover, oral administration of saffron ethanolic extracts showed a significant ($P<0.05$) decrease in edematous paw volume in a time-dependent manner. SEE (800 mg/kg) produced an anti-inflammatory activity 1 h after administration and continued until the end of the experimentation, with the most prominent inhibition of 77.33% followed by PEE (70.50%) at the 4th h of study. LEE and CEE exhibited moderate but significant ($P<0.05$) potential with the percentage edema inhibition of 53.29% and 47.47%, respectively.

Table 4.24: Anti-inflammatory effect of *C. sativus* ethanolic extracts

Group	Dose (mg/kg)	Mean edema volume (ml)			
		1 h	2 h	3 h	4 h
Saline	1 ml/kg	0.47 ± 0.03	0.56 ± 0.03	0.69 ± 0.04	0.76 ± 0.03
DP	10	0.25 ± 0.02***	0.17 ± 0.01***	0.08 ± 0.01***	0.04 ± 0.01***
CEE	800	0.43 ± 0.03	0.37 ± 0.03***	0.30 ± 0.01***	0.25 ± 0.03***
LEE	800	0.37 ± 0.03**	0.31 ± 0.02***	0.26 ± 0.01***	0.20 ± 0.03***
SEE	800	0.26 ± 0.01***	0.19 ± 0.02***	0.14 ± 0.01***	0.07 ± 0.01***
PEE	800	0.29 ± 0.02***	0.23 ± 0.02***	0.17 ± 0.01***	0.11 ± 0.01***

Each value is represented as mean ± S.D. Where, *p<0.05, **p<0.01, ***p<0.001 statistically significant relative to control (saline)

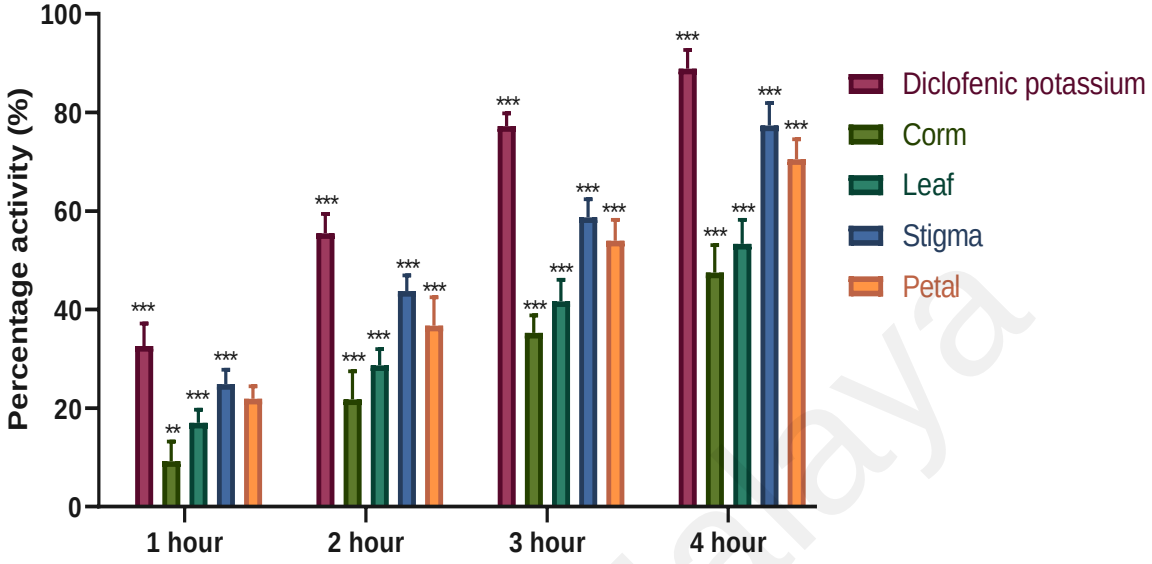


Figure 4.19: Percentage edema inhibition of C. *sativus* ethanolic extracts at selected time period in mice. Each value is represented as mean ± S.D. Where, *p<0.05, **p<0.01, ***p<0.001 statistically significant relative to control (saline)

4.5.3 Anticoagulant assay

The blood clotting activity of saffron extracts was investigated using the capillary tube method. The results presented in figure 4.20 show the effects of oral administration of saffron extracts and aspirin on coagulation time in mice. As a reference drug, the anti-coagulant aspirin (10 mg/kg) significantly increased blood clotting time (108.5 ± 8.59 s) compared to the control group, saline (38.33 ± 4.92 s). SEE was the major identified anti-coagulant extract showing prominent and significant anticoagulant effect with coagulation time of 101.66 ± 7.20 s followed by PCC (86.50 ± 6.89 s), respectively. LEE, however, had moderate inhibitory effect on coagulation activity with a clotting time of 66.83 ± 6.17 s. CEE, on the other hand, was unable to prolong the blood clotting time (42.83 ± 6.27 s) and showed an almost equal response to saline representing neutral effect of the selected extract.

4.5.4 Antidepressant activity

The antidepressant activity of orally administered saffron ethanolic extracts was tested by forced swimming test and findings in the form of immobility time are graphically depicted in figure 4.21. Positive control group administered with drug Fluoxetine HCl produced strong antidepressant effect (41.33 ± 4.71 s) at the concentration of 10 mg/kg against the negative control, saline (141.16 ± 6.40 s). Furthermore, saffron ethanolic extracts significantly attenuated immobility time in mice when compared with the saline-treated control group. At the dose of 800 mg/kg, PEE represented itself as a potential antidepressant candidate showing significant reduction in immobility time (69.66 ± 7.63 s) with respect to control and equivalent to the standard drug followed by SEE (76.66 ± 6.56 s). CEE significantly declined mean time spent by mice in immobile state (96.50 ± 6.28 s) signifying moderate antidepressant effect, whereas LEE exhibited mild activity (106.83 ± 6.24 s) by significantly attenuating immobility time with respect to serine, but not equivalent to fluoxetine.

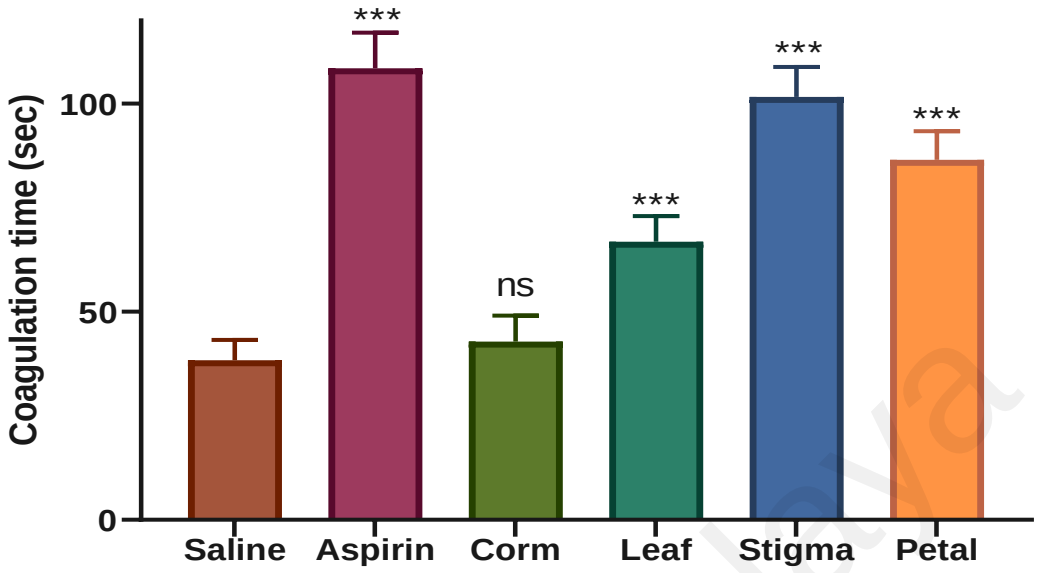


Figure 4.20: Anticoagulant effect of *C. sativus* ethanolic extracts. Each value is represented as mean ± S.D. Where, *p<0.05, **p<0.01, ***p<0.001 statistically significant relative to control (saline)

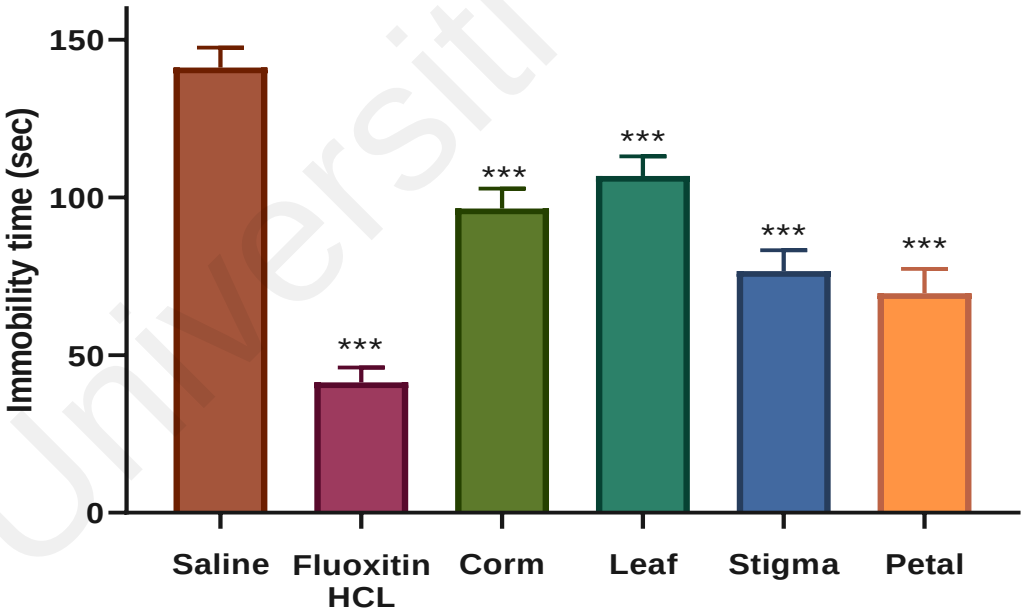


Figure 4.21: Antidepressant effect of *C. sativus* ethanolic extracts. Each value is represented as mean ± S.D. Where, *p<0.05, **p<0.01, ***p<0.001 statistically significant relative to control (saline)

4.5.5 Antidiabetic activity of saffron

4.5.5.1 Anti-hyperglycemic potential in glucose loaded diabetic animals

The changes in the blood glucose level of normal and diabetic mice after 0 to 120 min of extract treatment is illustrated in figure 4.22. Tabulated results revealed a significant increase ($p<0.05$) in blood glucose level of STZ induced diabetic mice as compared to the normal control group. Blood glucose reached the highest level at 60 min after oral treatment of glucose in diabetic control group, that was maintained until 120 min. Treatment of diabetic mice with SEE and PEE showed a significant decrease ($p<0.05$) in blood glucose level from 285 mg/dL to 131 mg/dL and 291 mg/dL to 150 mg/dL, respectively at various time durations. CEE group after treatment for 2 hours gradually decreased plasma blood glucose level from 272 mg/dL to 179 mg/dL while LEE treatment shower poor response by reducing elevation in glucose level from 280 mg/dL to 186 mg/dL after 0, 30, 60 and 120 min of saffron treatment.

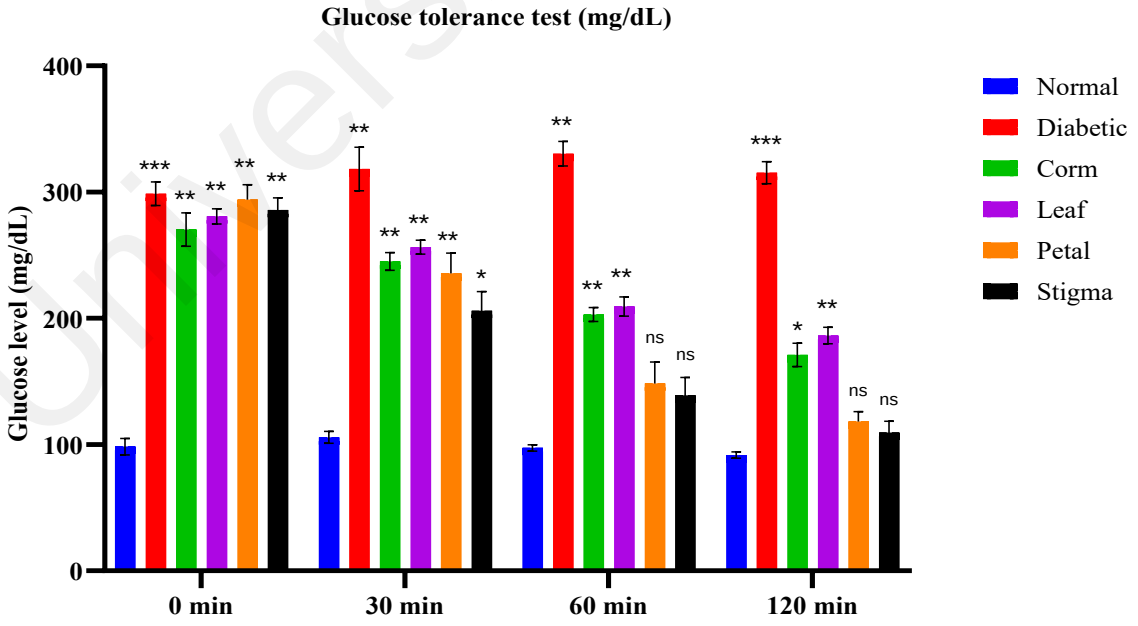


Figure 4.22: Blood glucose concentration in glucose loaded diabetic mice. Each value is represented as mean ± S.D. Where, *p<0.05, **p<0.01, ***p<0.001 statistically significant relative to control

4.5.5.2 Blood glucose level

The effect of saffron ethanolic extracts on the fasting blood glucose levels of diabetic mice is presented in figure 4.23. A significant ($p<0.05$) elevation in initial blood glucose levels of all diabetic mice was observed after induction with STZ as compared to the normal control mice. During the experimental study, higher significant difference ($p<0.05$) in blood glucose concentration was reported in the diabetic group in comparison with the normal control group. Oral administration of SEE caused significant ($p<0.05$) reduction in blood glucose levels at the end of the first, second and third week compared to the positive control group showing glucose level of 171, 138 and 118 mg/dL, respectively. Likewise, PEE significantly declined ($p<0.05$) the increase in blood glucose concentration after the first week till 21 days with glucose levels of 194, 153 and 129 mg/dL respectively. Oral treatment with CEE and LEE resulted in a lesser anti-hyperglycemic effect showing blood glucose level of 268, 210, 176 and 160 mg/dL for CEE and 258, 236, 197 and 183 mg/dL for PEE during the 21 days experiment. It indicates that the decrease in blood glucose level during treatment was more detectable in ethanolic extracts of floral parts than vegetative parts of saffron.

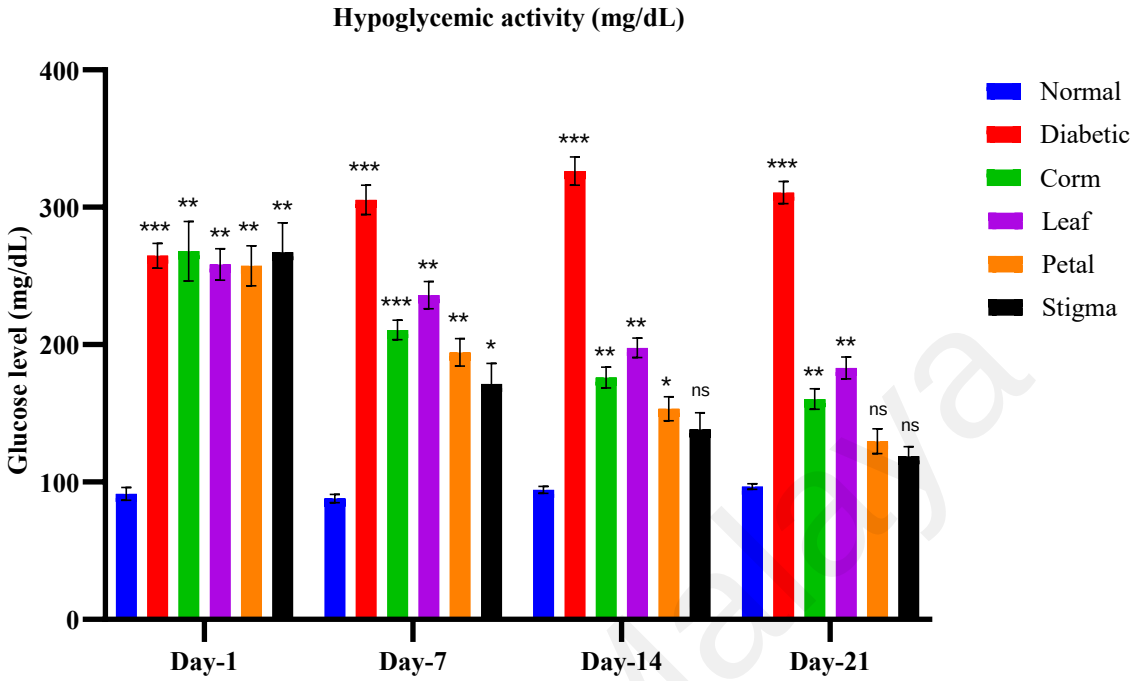


Figure 4.23: Hypoglycemic activity of *C. sativus* ethanolic extracts in diabetic mice. Each value is represented as mean ± S.D. Where, *p<0.05, **p<0.01, ***p<0.001 statistically significant relative to control

4.5.5.3 Serum concentrations of total lipids, triglycerides, and total cholesterol

Serum concentrations of LDL, HDL, TG and TC in diabetic and nondiabetic groups of mice was recorded and presented in figure 4.24. There was a significant ($p<0.05$) increase in serum levels of LDL, TG and TC but significant decrease in HDL level of diabetic control compared to normal control. According to the results, treatment of SEE and PEE to diabetic mice reverted the level of above serum lipid markers towards the normal control mice after 21 days. In addition, treated diabetic mice with CEE showed prominent effects in decreasing the LDL contents. Among all the tested extracts, LEE administration was less effective in restoring the serum levels of LDL, HDL, TC and TG in diabetic mice.

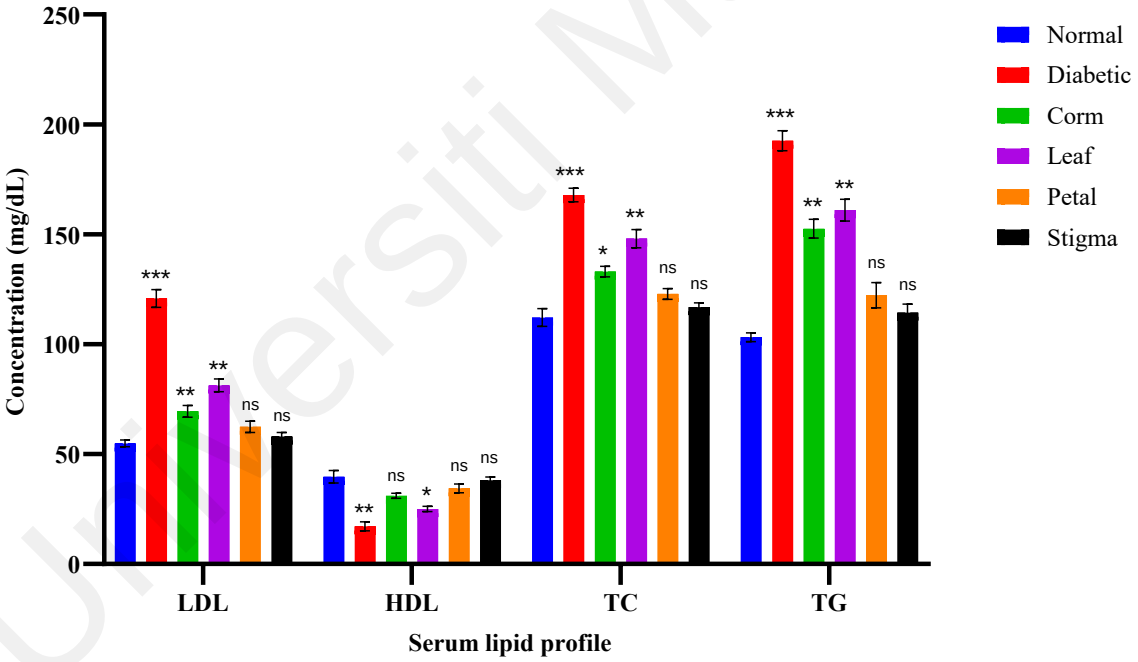


Figure 4.24: Protective outcome of *C. sativus* ethanolic extracts on serum lipid profile. Each value is represented as mean ± S.D. Where, *p<0.05, **p<0.01, ***p<0.001 statistically significant relative to control

4.5.5.4 Liver functions assay

The levels of serum AST, ALT, and ALP as indicator of liver functions in the mice are given in figure 4.25. The data revealed that STZ induced diabetic mice showed significant ($p<0.05$) elevation in ALT, AST and ALP concentration as compared to normal control mice. Administration of saffron ethanolic extracts restored the concentration of above altered parameters towards the normal control. However, the decreased in serum concentrations of liver biomarkers were more pronounced with SEE and PEE as compared to CEE at the respective dose. Conversely, LEE treated diabetic mice showed significantly ($p<0.05$) higher level of ALT and ALP when compared with the normal control group.

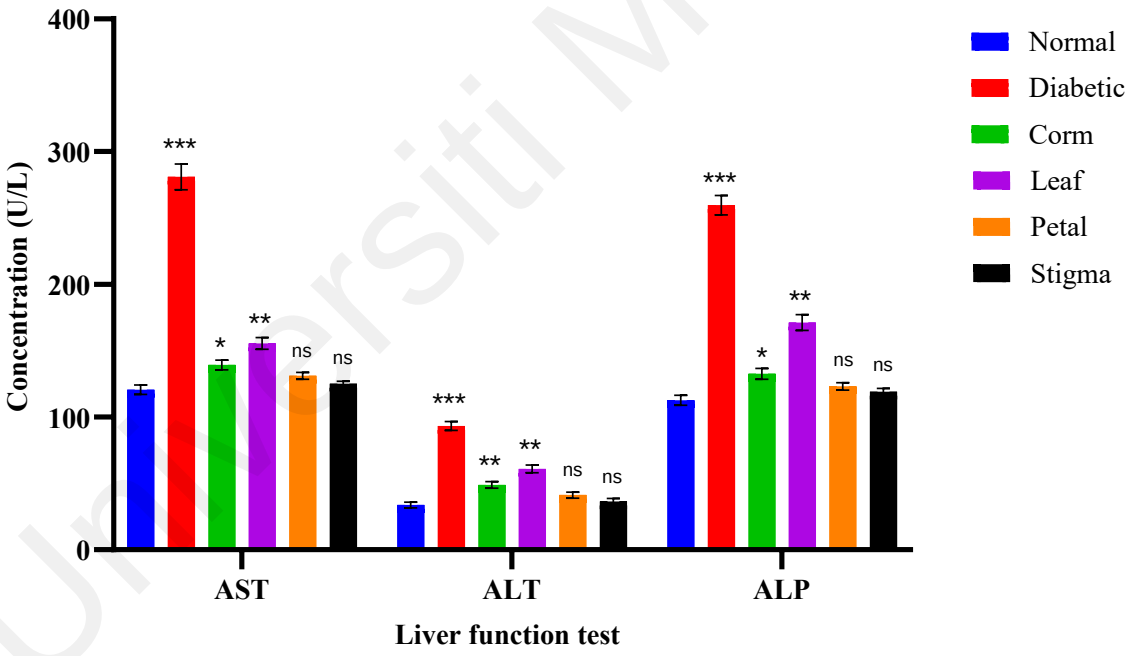


Figure 4.25: Protective outcome of *C. sativus* ethanolic extracts on serum profile of liver. Each value is represented as mean ± S.D. Where, *p<0.05, **p<0.01, ***p<0.001 statistically significant relative to control

4.5.5.5 Kidney functions assay

The measured concentrations of serum creatinine, BUN, and UA as indicator of renal functions in the mice are shown in fig. 4.26. Results showed that in comparison to control mice, serum creatinine, BUN and UA levels were significantly ($p<0.05$) higher in STZ induced diabetic mice as expected. Among the tested saffron extracts, SEE exhibited lowest values of all kidney biomarkers showing no significant difference to the control group followed by PEE. Likewise, insignificant alterations in serum kidney biomarkers were noticed in CEE treated mice compared to normal control group. On the contrary, LEE treated diabetic mice showed significantly ($p<0.05$) higher level of the above parameters compared with the normal control.

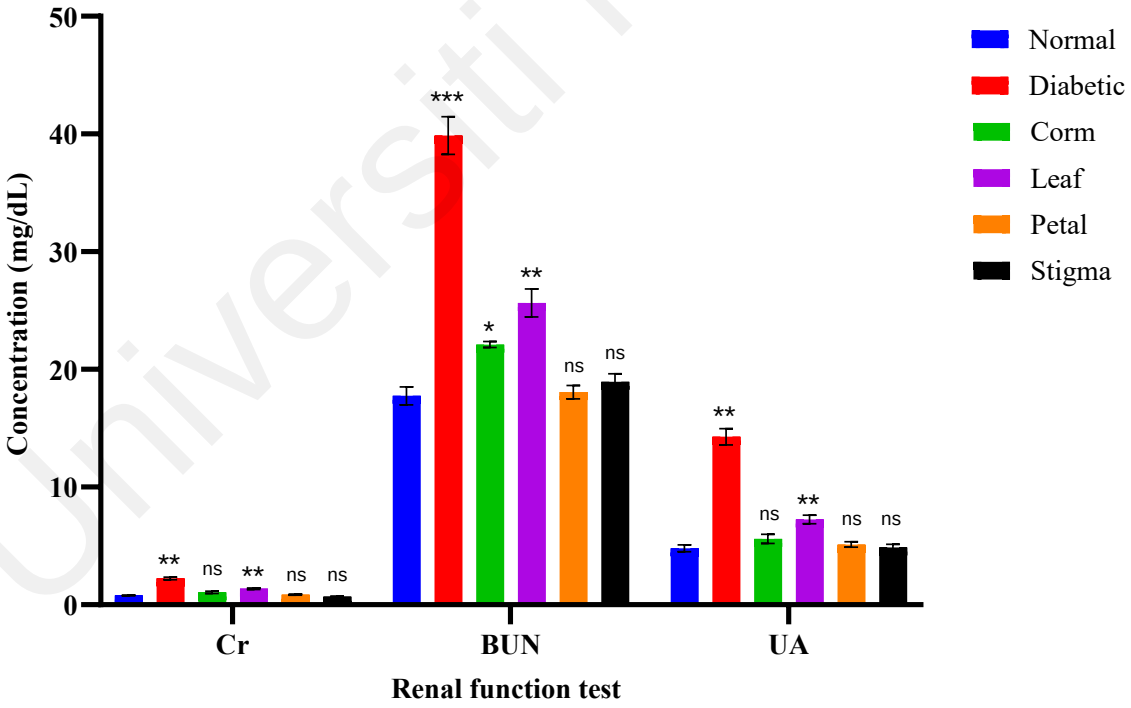


Figure 4.26: Protective outcome of *C. sativus* ethanolic extracts on serum profile of kidney. Each value is represented as mean ± S.D. Where, *p<0.05, **p<0.01, ***p<0.001 statistically significant relative to control

4.5.5.6 Histopathological study

Pathological examinations of pancreas and liver sections of normal control, diabetic control and SEE treated group are depicted in figure 4.27. Light microscopic examination of pancreas sections from normal control group revealed normal morphology and histological structure (figure 4.27A). However, pancreas of mice from STZ induced diabetic group showed acute distortion, inflammatory cell invasion and reduction of β cell mass (figure 4.27B). On the contrary, the administration of SEE treated group expressively restored the normal structure of pancreas by showing no detectable abnormalities and alterations (figure 4.27C).

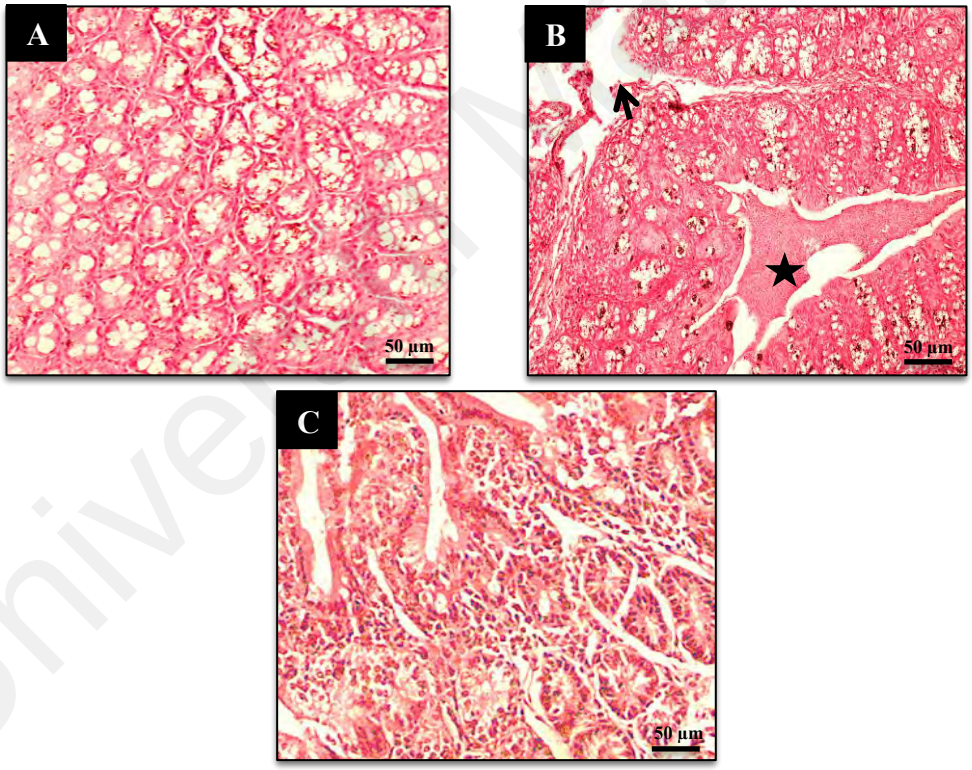


Figure 4.27: H and E staining of pancreatic tissues of mice at 40X magnification. (A) Normal control with normal pancreatic structure; (B) STZ induced diabetic mice with damaged pancreatic tissue, reduced β cell mass and inflammatory cell invasion; (C) SEE treated diabetic mice with a structure comparable to normal group. Here, sign ↑ represents inflammatory lobules and inflammatory cells whereas sign★ represents a reduction in β cell mass

Correspondingly, the photomicrographs of the liver sections displayed normal hepatocyte morphology with no evidence of inflammation and necrosis in normal control group as shown in figure 4.28A. In STZ-diabetic control group hyperammonemia, lobular inflammation, abnormal patterns with lymphocyte infiltration along with cytoplasmic degeneration at various spots were observed (figure 4.28B). However, SEE treated diabetic group reversed the pathological alterations towards normal mice with slight lymphocyte infiltration in liver (figure 4.28C).

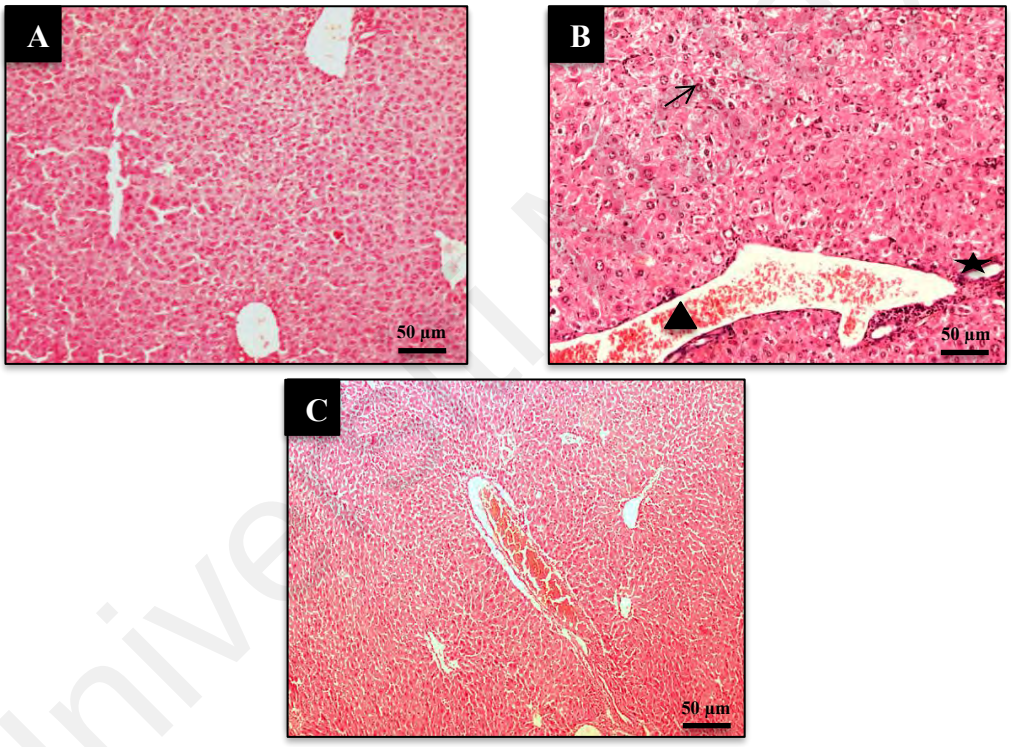


Figure 4.28: H and E staining of liver tissue of mice at 40X magnification. (A) normal control with no significant change; (B) STZ induced diabetic mice representing liver tissue damage, necrosis at many spots and lymphocyte infiltration; (C) SEE treated diabetic mice comparable to the normal with slight inflammatory invasion and tissue regeneration. The sign ↑ represents lobular inflammation and inflammatory cells invasion, ★ represents hyperammonemia, and ▲ represents cellular degeneration

DISCUSSION

Saffron corms obtained from the open fields are seriously infected by numerous pathogens, thus making it a challenge for culturing *in vitro*. Therefore, proper sterilization procedure for *in vitro* regeneration of saffron corms is very essential. In the present study, preliminary trials were undertaken to ensure the best sterilization protocol against surface and endogenic contamination. Individual treatment of sterilant was not enough to overcome the contamination rate. However, combining effect of sterilant showed a significant increase in the production of aseptic and viable cultures. Among all the sterilization protocols used, sterilization of corms for 2-3 min with 70% ethyl alcohol, washing with Tween 20 for 30 min followed by 40% clorox for 15 min and 0.20% $HgCl_2$ for 10 min showed the highest percentage for aseptic cultures (93%) and survival rate (93%). Higher concentration of $HgCl_2$ (0.30%) and clorox (50-60%) ensured maximum aseptic cultures (96%) but decreased explant viability (70%) and resulted in necrosis.

Sterilization of saffron explants treated with ethyl alcohol, NaOCl, H_2O_2 and $HgCl_2$ in different concentration is already documented in the literature (Cavusoglu et al., 2013; Devi et al., 2011; Ebrahimzadeh et al., 2000; Loskutov et al., 1999; Rajabpoor et al., 2007; Vahedi et al., 2015). Yasmin *et al.* (2013) was able to get 94% clean aseptic saffron cultures by using the combination of systemic fungicides with 50% clorox for 10 min followed by a dip in 1.6% $HgCl_2$ for 5 min. In another experiment, corms were treated with different sterilization methods and found 100% aseptic cultures by washing with 70% ethyl alcohol for 5 min followed by 50% clorox and 7% H_2O_2 for 15 min each (Cavusoglu et al., 2013). Similarly, Sivanesan *et al.* (2014) obtained 100% contaminant-free cultures of *C. vernus* corms after applying 2.0% NaOCl for 10 min prior to washing with 0.01% $HgCl_2$ for 15 min.

The usage of saffron corms as an explant is a common modality in tissue culture studies due to the presence of meristematic nodes. Therefore, sterilized corms were cultured on two media such as MS medium and Gamborg medium to find out the influence of medium composition and growth regulators on sprouting response. In the studies conducted earlier, the bud sprouting efficiency of cytokinins and auxins was very less when used as individual and the effect was more significant in combination (Salwee & Nehvi, 2014), therefore a combination of auxin with cytokinin were used. Bud sprouting efficiency of saffron per corm was higher in MS medium as compared to Gamborg medium under all kinds of auxin (NAA, IAA) and cytokinin (Kn, BAP). Highest bud sprouting response (100%) and number of sprouted corms (4.90) were achieved when corms were cultured on MS medium fortified with IAA (1 mg/l) in combination with BAP (2 mg/l). Similar results with no significant difference were observed with IAA (1 mg/l) and BAP (4 mg/l), whereas corms cultured on MS medium containing IAA (2 mg/l) and BAP (2 mg/l) showed lowest sprouting response (40%) and sprouted number (1.40). On the other hand, Gamborg medium gave highest bud sprouting response and sprouting number of 90% and 3.70 on 1 mg/l IAA + 4 mg/l BAP, respectively. Absence of PGRs in the medium showed the least sprouting percentage (40%) and sprouted corms (0.90) followed by medium containing NAA (2 mg/l) in combination with Kn (2 mg/l).

Salwee & Nehvi (2014) reported maximum bud sprouting response of 85.94% with MS medium enriched with NAA (0.5 mg/l) and BAP (1.5 mg/l) and failed to produce any sprouting in the absence of PGRs. Addition of PGRs to the media has a significant impact on the enhancement of sprout number. However, higher concentration has been shown to have inhibitory effect on the sprout number (Salwee & Nehvi, 2014). In another experiment, corms initiated bud sprouting in MS fortified with BAP (6 mg/l) in combination with all the

concentrations of 2,4-D (1,2 and 4 mg/l) tested. However, only the shoots produced in the medium having BAP (6 mg/l) with 2,4-D (2 mg/l) resumed growth (Devi et al., 2011).

As shoot development requires inclusion of cytokinin in the medium, hence corms that showed 100% success in IAA (1 mg/l) and BAP (2 mg/l) containing medium, were transferred to MS medium containing IAA (1, 2 mg/l) plus different concentration of BAP (2, 4, 6 mg/l) or Kn (2, 4, 6 mg/l). After 5 weeks, direct multiple shoot primordia in the form of bunches of shoots appeared at the base of sprouted buds. Some of the single initiated shoots persisted throughout the growth period and turned yellow after reaching a certain length. Almost all conditions inducted shoot primordia but showed varied response. Highest number of shoot primordia (5.60) was achieved in MS fortified with IAA (1 mg/l) and BAP (4 mg/l). However average length of shoot primordia was higher (2.33 cm) in MS medium containing IAA (1 mg/l) and BAP (6 mg/l). Inclusion of Kn in the medium showed poor response towards induction of multiple shoot primordia. Whereas the least number of multiple shoot primordia (1.20) was attained in MS basal medium. Induction of multiple shoot primordia by media enriched with high concentration of BAP is consistent with the findings made by Devi et al. (2011). In another experiment, the interaction effect of NAA (0.2 mg/l) plus BAP (6.5 mg/l) was significantly more profound showing highest number of shoot primordia (24) compared to the individual effect of each hormone (Yasmin et al., 2013).

Further sub culturing of shoot primordia on MS medium containing IAA (0.5 and 1 mg/l) in combination with BAP (2, 4 and 6 mg/l) or Kn (2, 4 and 6 mg/l) resulted in multiple shoot proliferation. In the present study, shoot multiplication increased with an increase in concentration of BAP from 2-6 mg/l in combination with IAA (0.5 mg/l). Among different concentration of IAA and BAP used, MS supplemented with IAA (0.5 mg/l) and BAP (6

mg/l) was most superior to enhance shoot number (6.40) and shoot length (4.38 cm) followed by combine treatment of IAA (0.5 mg/l) and BAP (4 mg/l) where the mean shoot number and shoot length was found to be 5.06 and 2.93 cm per culture, respectively. On the other hand, Kn was only effective at a concentration of 6 mg/l in combination with IAA (0.5 mg/l) giving a mean shoot number of 4.13 per culture. Devi et al. (2011) observed highest shoot number (10.25) in combination with BAP (6 mg/l) and NAA (0.2 mg/l), whereas the highest shoot length (7.58 cm) was attained at BAP (6 mg/l) plus NAA (1 mg/l).

Auxins alone or in combination with other PGRs, play key role in rooting (Zeybek et al., 2012) and cormlet formation of saffron (Mir et al., 2010; Zeybek et al., 2012). Therefore, explants that produced multiple shoots, were cultured in MS medium containing IAA (0.5 mg/l) with different concentrations of sucrose and GA_3. Development of daughter corms initiated after 3 to 4 weeks of culture in the form of swelling at the base of the shoots. Different concentration of sucrose and GA_3 showed significant differences in their cormlet number and cormlet weight when added to MS medium supplemented with IAA (0.5 mg/l). Significantly, maximal cormlets number (4.33) and cormlet weight (0.58 g) were obtained in medium fortified with GA_3 (4 mg/l) and 4% sucrose. MS medium with no PGR and sucrose was ineffective for inducing cormlet formation.

Presence of sucrose in culture media is important for differentiation of cormlet as in earlier studies no daughter corm was produced in sucrose free media and only 0.29 per shoot (0.18 g average fresh mass) formed in mannitol containing media (Sharma et al., 2008). Higher concentration of GA_3 showed significant effect on cormlet proliferation and weight with no significant effect on root proliferation and length. According to data, media with 2% sucrose

and GA_3 (2 mg/l) gave the highest root number (5.2) and root length (13.10 cm) per root formed explants.

Devi et al. (2011) used varied concentration of growth retardants such as abscisic acid (ABA), paclobutrazol (PBZ), chlorocholine chloride (CCC) and ancymidol for cormlet production in saffron and found higher cormlets (86.74%) and mean cormlet number (4.33) per corm explant in media supplemented with PBZ (1 mg/ml). Mir et al. (2014) also reported maximal cormlet number (10) and weight (1.54 g) on MS media containing PBZ (1.5 mg/l) in combination with NAA (0.5 mg/l) and BAP (2 mg/l). In another experiment, Cavusoglu et al. (2013) cultured saffron explants in MS medium with 1 and 2 mg/l concentration of IBA or IAA and obtained highest rate of cormlet production (76.7 %) and mean cormlet number (1.74) per corm from MS plus IAA (1 mg/l). In contrast, maximum initiated corms (0.52) and number of corms (1.04) from direct regenerated shoots were produced in IBA (1 mg/l) containing media. However, initiated roots and number of roots favored high concentration of IBA (2 mg/l) (Zeybek et al., 2012).

According to the achieved results, lower temperature of 18 °C, bigger sized of planted corms and manure application favored the growth and yield of saffron. As discussed, temperature plays a key role in saffron flower induction and appearance. Several studies have illustrated the effect of temperature on flower formation in saffron (Gresta et al., 2009; Molina et al., 2004; Molina et al., 2004; Molina et al., 2005; Wang et al., 2021). However, they did not indicate the single or interaction effect of temperature on flowering and vegetative yield of saffron.

In the present study, the highest FFW, FDW, SFW, SDW, DCN and DCW were achieved when corms were grown at 18 °C and significant reduction in the growth parameters was

observed with increasing the temperature up to 23 °C. In saffron, flower initiation occurs during late spring as the rising temperature reach above 20 °C, whereas flower starts to emerge as the temperature drops below 16 °C (Kumar et al., 2009). According to some authors, rainy seasons, warm summers and mild winters are the optimal climatic conditions for saffron (Fernandez, 2004; Mollafilabi, 2004), even though some saffron are reported to show tolerance at -18 °C (Mollafilabi, 2004), as well as high temperatures up to 40 °C (Gresta et al., 2009). Molina et al. (2004), reported that maximum number of flowers were attained with corms incubated at 25 °C for more than 55 days prior to planting at 17 °C. However, a long exposure to 25 °C exceeding 150 days showed a considerable decrease in flower number.

The present study demonstrated that the interaction of temperature with harvesting year, mother corm size, type of fertilizer and planting depth had no significant effect on the floral and vegetative yield components of saffron. However, lower temperature of 18 °C irrespective of any studied factor was more suitable for higher floral yield and daughter corms formation. These results are in accordance with the findings of Koocheki et al. (2011) whereby highest flowering response and maximum number of vegetative buds were attained with mother corms incubated for 90 days at 27 °C followed by forcing at 17 °C for flower emergence. These findings suggest that lower temperature between 17 °C to 18 °C is the optimum temperature required for better saffron growth and yield components.

As discussed, saffron is a sterile plant that propagates solely through corm. Thus, one of the most important ways to increase saffron yield is the farm management improvement, especially by choosing high quality saffron corms. The effect of the mother corm size on the quantitative parameters of saffron has been widely studied. According to Renau-Morata et

al. (2012), this agricultural factor determines the presence or absence of flowers, given that if the corms do not attain their normal size, they only produce leaves. Also, saffron corm needs to be above a minimum size to produce flowers as flowering has a positive correlation with the average weight of the corms planted (Gomez et al., 1987) and therefore, cultivation of large sized corms gives more yield than smaller ones (Douglas et al., 2014).

In the present study, maximum amount of flower and replacement corm yield for all variables were obtained significantly with bigger corms of 15 g compared to smaller corms of 10 g. Bayat et al. (2016) concluded that using 10 g or bigger corms of saffron increased flower fresh weight and flower number than using corms of 6 g or smaller corms. On the contrary, very big corms reduce the number of flowers per corm possibly due to aging (Mashayekhi et al., 2006). Therefore, intermediate corms of 8-16 g in weight and 2.5-3.5 cm in dimension are highly recommended for commercial production of saffron (Kumar et al., 2009).

In our study, the interaction of mother corm and harvesting year demonstrated that corm class of 15 g showed significantly higher FFW, FDW, SFW, SDW and DCN than corm class of 10 g irrespective of harvesting season. On the other hand, the interaction between mother corm weight and planting depth had no significant effect on all the six variables of saffron. However, the quantitative yield of saffron to bigger corms grown at low depth was more remarkable. These studies show that corms with bigger size might have a higher physiological ability for early flower initiation and due to more food reserves, increase the percentage of flower emergence, and ultimately improve quantitative yield of saffron (Khorramdel et al., 2015).

The application of fertilizers and organic manures is routinely used in agriculture to enrich the soil with an adequate quantity of essential nutrients for better yield of saffron. In the present study, application of cattle manure to soil showed a significant increase in all of the quantitative indices of saffron as compared to chicken fertilizer. Similarly, the interaction of type of fertilizer and harvesting year demonstrated that soil containing cattle manure increased the quantitative yield of saffron as compared to chicken fertilizer irrespective of harvesting season. On the other hand, the interaction between the type of fertilizer and planting depth showed no significant effect on saffron growth attributes. However, the quantitative yield of saffron was more favorable in soil containing manure with planting depth of 16 cm. These results are in accordance with the findings of Jahan & Jahani (2007), whereby the effect of different combinations of chemical and organic fertilizers on saffron production showed that cow manure had maximum effect on number of flowers, and therefore more stigmas. They further remarked that animal manures were more effective than chemical fertilizers in yield enhancement of saffron.

Under Iranian conditions, during the first year of experiment, the application of composted cattle manure and chemical fertilizers showed no significant effect on flower number and dried stigma yield. However, a significant increase in the flower number and dried stigma yield was observed during the second year of experiment (Koocheki & Seyyedi, 2015). Likewise, Yarami & Sepaskhah (2015) studied the effect of cow manure as well as irrigation water salinity and different planting methods on saffron yield, and reported that higher cow manure application (60 Mg ha^{-1}) increased the yield of saffron by 20% and 25% in the first and second seasons, respectively. Thus, it can be concluded that manure application to soil favors both the floral and vegetative parameters of saffron.

It has been found that saffron corms are more exposed to temperature fluctuations with decreasing soil depth (Galavi et al., 2008). On the other hand, flowers emergence and leaves formation may be delayed or not occur at greater planting depth (Koocheki & Seyyedi, 2019). Hence, selecting the best sowing depth is very crucial in saffron for better yield of the crop. With regard to the planting depth, no significant effect on all the variables related to flowering (FFW, FDW, SFW and SDW) and daughter corms (DCN and DCW) was observed in the present study. These findings are in accordance with the results of Negbi et al. (1989) who confirmed that planting depth has no effect on saffron growth and flowering. However, according to De Juan et al. (2009) planting corms at a depth of 10 cm favored quantitative yield of saffron stigma as compared to planting depth of 20 cm. On the contrary, low planting depth is not recommended for saffron due to the cold sensitivity (Benschop, 1993), although Rees (1988) concluded that saffron can tolerate severe frosts.

With respect to the harvesting season, saffron growth indices were significantly affected in the present study. Growth indices such as FFW, FDW, SFW, SDW and DCN were significantly higher at the end of the first harvesting season as compared to second year. The reduction during the second year of the experiment might be due to the production of small daughter corms by mother corms during the first harvesting season. In other words, small daughter corms produced would have low capability for flower formation in the second year (Gresta, F et al., 2008). Similar findings were reported by Koocheki & Seyyedi (2015), whereby flower number and dried stigma yield were significantly reduced by the cultivation of mother corms weighing >12 g during the second year of the experiment compared with the first year.

It is known that gamma radiation is tremendously used as a physical mutagen in mutation breeding and is found to be very applicable for persuading disparity in plant system. This consequence can occur both impulsively by nature and artificially by mutagens. The relationship between doses of gamma radiation and growth of the irradiated plants has been determined by studying the vegetative and reproductive characteristics of saffron. Also, the positive and negative consequences of gamma irradiation were assessed by comparison to non-irradiated plants in saffron. In all, vegetative and floral characteristics of saffron were significantly retarded with increasing doses of gamma radiation.

In the present study, higher doses of gamma rays significantly reduced survival and sprouting rate in saffron corms showing the maximum percentage of corms survival (100%) and sprouting (100%) in low doses (10 Gy and 20 Gy) compared to the higher doses (30 Gy and 40 Gy). The reduction in survival rate and sprouting percentage of saffron corms attributes to the genetic damage due to gene mutations and chromosomal aberrations (Khan et al., 2011). Therefore, increasing frequency of chromosomal damage with increasing dosage is responsible for the reduction in plant growth and survival.

Some other studies also reported the negative effect of mutagens on the survivability of saffron corms (Khan, 2004; Nehvi et al., 2010). Corms subjected to 1 Gy, 2 Gy, 3 Gy, 4 Gy, and 5 Gy have been found to result in a survival rate of 98%, 99%, 90%, 72%, and 68%, respectively (Nehvi et al., 2010). Likewise, corms exposure from 0 to 15 Gy recorded maximum survival rate (100%), while 20 Gy, 25 Gy, and 30 Gy recorded corms survivability of 54%, 47%, and 20%, respectively (Khan, 2004). Low survival and germination percentage in response to increased doses of gamma rays have also been reported in chrysanthemum (Soliman et al., 2014), rose (Bala & Singh, 2013), ginger (Rashid et al., 2013), okra (Asare

et al., 2017), gladiolus (Sathyanarayana et al., 2019), safflower (Srivastava & Kumar, 2011), and sunflower (Hussain et al., 2017).

The saffron plants differed significantly in days to sprouting response among the various treatments. Untreated control corms sprouted earlier (43 days) compared to the irradiated corms. However, there was no significant difference in days to sprouting between untreated (43.37 days) and 10 Gy plants (46.87 days). The stimulating causes of radiation on sprouting may be associated with RNA activation, which occur during the initial stages of germination after irradiation of corms (Amir et al., 2018). According to Jan et al. (2011), the growth inhibition induced by higher doses is due to the higher psoralen production that results in inhibiting certain phytohormones without otherwise harming the crop.

The present study is in close conformity with the findings of Khan (2004) in saffron and Sathyanarayana et al. (2019) in gladiolus, whereby dosage of higher than 10 Gy resulted in slow growth and delayed sprouting of corms. Low level of mutagen itself is not responsible for the early sprouting of corms but it influences the activity of enzymes. Enzymes play an important role in plant metabolism to accelerate metabolic activities and consequently result in stimulating plant growth. Pandey & Gaur (1984) reported that corms irradiated with low dose showed reduction in sprouting time, elevation in O_2 uptake, stimulation of alpha-amylase enzyme before sprout emergence, and an increase in sugar content. On the other hand, higher doses delayed the sprouting time, reduced oxygen uptake, suppressed alpha-amylase activity, but induced a slight increase in sugar content.

Morphological variation in the leaf traits is a primary effect of mutagenic studies. In our study, a significant reduction in the number of leaves and leaf length was recorded with higher doses of radiation in treated corms. Maximum number of leaves per plant (5.93) and

leaf length (14.78 cm), respectively were reported in 10 Gy plants. Other workers also reported the stimulatory effect of lower doses and inhibitory effect of medium to higher doses on leaf number and leaf length (Singh & Anuj, 2013). The number of leaves and leaf length reduced gradually as the doses of gamma rays increased compared to the control plants in chrysanthemum (Kumari et al., 2013). According to Nehvi et al. (2010), 4 Gy and 5 Gy treatment was more beneficial for enhancing the number of leaves per plant and leaf length compared to control and lower doses (1 Gy, 2 Gy, and 3 Gy) in saffron.

In the present study, 40 Gy plants failed to produce true leaves directly from the shoot and formed cataphylls only. This abnormal development of leaves was most likely due to the physiological disturbances and/or chromosomal aberrations in 40 Gy treated plants (Bala & Pal Singh, 2013). Besides, irradiation mutagenic treatment negatively influenced plant height under all treatments as maximum plant height was noticed in control plants. These observations are in line with the results obtained by Jun et al. (2006) in saffron, Sathyanarayana et al. (2019) in gladiolus, Kumari et al. (2013) in chrysanthemum, Tshilenge-Lukanda et al. (2013) in groundnut, and Justin et al. (2012) in soybean. Reduction in plant height, leaf number, and leaf length may be due to the reduction in the number of vertical cell layers resulting in the shorter internodes, number of internodes, or any combination of these processes (Singh & Anuj, 2013). Nonetheless, the reduction in plant growth is also associated with the destruction or damage to apical meristem and temporary suspension of cell division or delay in mitosis due to gamma radiation (Amir et al., 2018). However, the reports of Ahamed (2019) in saffron and Emrani et al. (2012) in canola contradict these results and revealed that plant height elevated with the application of gamma rays. This may be due to the different genetic material and environmental conditions (Tshilenge-Lukanda et al., 2013).

In the present study, floral characteristics of saffron were investigated and showed that 10 Gy plants started flowering earlier (85 days) than the control plants (88 days). However, a significant increase in days to flowering occurred with increasing the intensity of radiation dose. This may be due to an indirect effect of radiation treatment via delayed sprouting and slower growth, or the harmful effects of gamma irradiation on phytohormones i.e, auxins and gibberellins, or the induction of photo-insensitivity following gamma rays (Ali et al., 2015).

Early flowering in lower doses (5 Gy and 10 Gy) and marked delay in floral development in higher doses (15 Gy to 30 Gy) was also observed by Khan (2004) in saffron and Bala & Singh (2013) in rose. Further, the 10 Gy dose increased the flowering period to 8.8 days compared to 7.9 days in the control, whereas higher doses shortened the harvesting period. According to an estimate, it takes around 45 to 55 min to collect 1000 crocus flowers, and an additional 100 to 130 min are required to remove the stigma for drying. Thus, an average of 370 to 470 h of labour is required to produce just 1 kg of this unique spice (Cardone et al., 2020). The extension of the flowering period is, therefore, of great practical value as it will reduce the intensive and concentrated labour required for flower picking and stigma separation. The variation in the flowering period may be due to the enhanced or inhibit activity of the genes involved in the flower development (Rekha & Langer, 2007).

In the present study, control plants showed good flowering ability, with the highest number of flowers formed per corm, whereas all the treated plants significantly reduced the number of flowers per corm. Furthermore, 40 Gy plants failed to produce any flower and remained in vegetative state. Given the random nature of mutation treatments to DNA, this is not unexpected. The reduction in the number of flowers per plant or flowering ratio with respect to gamma irradiation is also reported in ginger (Taheri et al., 2014) and ornamental

plants (Bala & Pal Singh, 2013). However, these results contradict the observations made by Jun et al. (2006), where gamma-treated plants from 10 Gy to 25 Gy showed a flowering ratio of 108% compared to the untreated plants (106%).

The same pattern of response was observed for flower blooming. Less than 50% of flowers bloomed to a complete flower under all treatments compared to the highest blooming percentage of untreated plants (87%). Generally, petal development can be classified into two main stages: a slow growth stage resulting from cell division and a rapid growth stage resulting from cell expansion. Normally, at the end of cell division, the petals are only 35–40% of their final size and further growth and unfolding is primarily a result of differential cell elongation (Sood et al., 2006). Also, flower petal tissue contains high invertase activity that plays an important role in determining the patterns of assimilate distribution in flowers. It also helps in providing metabolites and energy to growing tissues for flower opening and various other biosynthetic processes (Sood et al., 2006). Zare et al. (2002) reported that soluble and insoluble invertase decreased with increasing levels of gamma irradiation and doses higher than 10 kGy inactivated the activity of invertase. Therefore, gamma rays might have reduced and/or inactivated invertase enzyme that ultimately reduced the flower blooming percentage of gamma-irradiated plants.

With respect to the flower weight, the highest flower fresh weight (172.90 mg) and dry weight (36.37 mg) was recorded with plants exposed to 20 Gy. This stimulatory effect of low irradiation dose on flower yield may be due to improving the hormonal signaling network and anti-oxidative capability of the plant to protect against environmental stress (Hong et al., 2014). Moreover, it can induce a resistance mechanism to cope with DNA damage and stimulates the immune system (Dimova et al., 2008). On the other side, 30 Gy and 40 Gy

revealed an inhibitory effect on flower fresh and dry weight. The observed decrease in the flower weight with increasing levels of gamma rays may be attributed to the slow cell division, abnormal transport of nutrients, and disruption of metabolic activity due to apical meristem damage as a result of gamma radiation (Baig et al., 2015).

Similar results were also reported by Ahamed (2019), who showed maximum flower fresh and dry weight in saffron plants exposed to 5-15 Gy with a corresponding decline at 25 Gy. Similarly, radiation doses ranging from 1-5 Gy were associated with the maximum fresh weight of saffron flowers in both generations as compared to the untreated plants (Nehvi et al., 2010). Improvement of flower fresh and dry weight using low gamma irradiation dose followed by a decline at high dose, has been documented by many researches in different plants, such as rose (Baig et al., 2015), chrysanthemum (Bosila & Hamza, 2019), and orchid (Billore et al., 2019). On the contrary, Hussain et al. (2017) reported an elevation in fresh and dry weight of sunflower with higher levels of gamma rays.

Due to the reduction of saffron yield and productivity, new investigations have been carried out recently to improve the yield and quality of stigma through the selection of corm geographical origin (Cardone et al., 2019), breeding techniques (Shokrpour, 2019), agronomic and climatic conditions (Askari-Khorasgani & Pessarakli, 2019) as well as the use of biostimulants (Caser et al., 2019). In the present study, unlike other floral characters, stigma fresh weight and dry weight in response to 20 Gy was significantly highest compared to the control and other doses. Since stigma is the most principal part of saffron used as a spice, in perfumery and herbal medicines, increase in the stigma weight is directly linked with the improvement in the saffron yield. These results are in accordance with the findings of Ahamed (2019) whereby lower doses increased stigma weight up to 113% while higher

dose declined stigma weight for 97% of the control (100%). The same pattern of response was observed for stigma length showing a maximum value of 4.96 cm at 20 Gy and the reduction of this trait afterward. Mutations in a gene called *maple-willow* (m^w) causes abnormal organ fusion (adnation) in the stigma, calyx, and corolla of *Ipomoea nil* (Kajita & Nishino, 2009). This gene has also been shown to display pleiotropic effects on leaf morphology. Reduction in stigma length observed in the high gamma-irradiated plants might have resulted from m^w gene mutation as revealed in M2 generations of *Capsicum annuum* treated by caffeine (Aslam et al., 2017).

Prominent morphological variations in the floral shape and petal color were found in the gamma-irradiated plants. Perhaps the most interesting mutation from a commercial perspective was the appearance of flowers with four irregular dark purple petals, tetrafid stigma, two stamens in one of the 20 Gy plants compared with six normal-sized purple petals, trifid stigma, and three stamens in the control. Floral shape and color variations in the said radiation treatment may be attributed to gene mutations, chromosomal disturbance, changes in chromosome number, rearrangement of different histogenic layers, and/or altered biochemical pathways. The latter is responsible for enhancing or reducing the concentrations of one or more pigments by either producing new pigments or inhibiting their synthesis (Bala & Singh, 2013).

Flower pigments are composed of flavonoids such as flavonols, flavones, and anthocyanins. Alterations in the core structural genes or regulatory loci of the biosynthetic pathway of anthocyanin result in floral color variations (Pallavi et al., 2017). According to Ghanbari et al. (2019), the darker color of the flower attributes to the condition of conical cells and higher amount of total anthocyanin in the petals. It is also pertinent to mention that

floral shape mutation was detected in one plant, whereas more than 80% of the 20 Gy flowering plants showed petal color's variation. Various reports regarding the variation of flower color and shape due to mutagenesis showed that 55% of the records concerned changes in flower color and 15% in flower morphology of ornamental plants. Several workers reported the induction of floral color and shape mutations after radiation treatment in common zinnia (Pallavi et al., 2017), gladiolus (Kumari & Kumar, 2015), rose (Bala & Singh, 2013), chrysanthemum (Setia et al., 2020; Soliman et al., 2014), common bean (Borkar & More, 2010), periwinkle (Hoda, 2014), orchid (Aloysius et al., 2018), coneflower (Oates et al., 2013) and wishbone flower (Suwanseree et al., 2011).

Normally, after flower formation, the meristematic tissue induction in mother corms stimulates the production of new corms or daughter corms (Koocheki & Seyyedi, 2019). The growth of daughter corms and gradual deterioration of mother corm lasts throughout the vegetative growth phase, which eventually develops the life cycle of saffron as a perennial plant (Seyyedi et al., 2018). In the present study, the total number and weight of daughter corms were favorably stimulated by low doses resulting in the maximum number of daughter corms (5.25 and 5 corms per mother corm) and daughter corms weight (3.74 g and 4.07 g) at 20 Gy and 10 Gy, respectively compared to the control. The production of more number of corms is one of the most economically important saffron attributes (Agayev et al., 2009).

In the present experiment, an increase in radiation dose over 20 Gy appeared to be deleterious. The favorable influence of these particular gamma rays might be expected because of the previously mentioned stimulating responses reflecting on the vegetative growth attributes of saffron. According to Kumari & Kumar (2015), the variations in the number of progeny corms may be attributed to the fact that higher doses disturb the plant

physiology which in turn affect photosynthesis and root system resulting in the improper growth of the plants by hampering root system thus, adversely affect the daughter corm production.

These results substantiate the findings of Ahamed (2019) who reported an elevation in daughter corm yield up to 125% and 117%, of control at lower doses (5 Gy and 15 Gy) that reduced to 97% of control at a higher dose (25 Gy). Significant increase in daughter corm number, weight, and size at 20 Gy followed by reduction at higher doses of 40 Gy and 80 Gy were obtained in gladiolus (Moustafa et al., 2018). Similarly, maximum corm number per plant (3) and per ha (141750) was recorded at a low dose (15 Gy) in another study on gladiolus cultivars (Devi & Fatmi, 2019). Krishnan et al. (2003) reported that number of bulbs size and weight in tuberose decreased with increase in irradiation treatment. In contrast, Jun et al. (2006) observed reduction in saffron cormlets number and weight under all levels of radiation treatment (5-25 Gy).

Herbal products are usually regarded as safe or of low toxicity based on their long history of use for the treatment of several ailments (Salmerón-Manzano et al., 2020). Nevertheless, latest reports have revealed the adverse effects of many of these products used in the traditional medicine (Ahmed et al., 2018; Al-Ghadeer & Al-Amry, 2021). Since, safety is a major concern with the use of herbal medicinal products, toxicity studies are necessary to ascertain the safety profile of the plant.

In the present study, the selection of 80% ethanol and/or ethanolic extracts was chosen due to their efficiency against cancer (Amin et al., 2011), blood pressure (Fatehi et al., 2003), inflammation (Akbari-Fakhrabadi et al., 2019) and nociception (Hosseinzadeh & Younesi, 2002). None of the orally administered saffron extracts (2000 mg/kg) caused any mortality

or prominent behavioral abnormalities in mice during the 14-days acute toxicity study. In literature, toxicological reports regarding saffron safety are not uniform. Iranshahi et al. (2011) assessed the toxicity level of 800 mg/kg SEE, PEE, stigma aqueous extract (SAE), and petal aqueous extract (PAE) and found no toxicological signs on mice. In another study, maximum non-toxic dose of SEE and PEE was reported as 2 and 8 g/kg, respectively (Hosseinzadeh & Younesi, 2002). Furthermore, LD_{50} values of intraperitoneal administered petal and stigma extracts in mice were 6 and 1.6 g/kg, respectively (Escribano et al., 1996). Similarly, no mortality of mice was reported within 2 days of study with high oral and intraperitoneal doses (3 g/kg) of active constituent of saffron, crocin in mice (Hosseinzadeh et al., 2010).

Changes in the body weight and water/feed consumption are used as an indicators of general health status of experimental animals. Increase or decrease in the body weights of animals is linked with the toxicity of drugs (Dongmo et al., 2019). Similarly, feed intake is controlled by many complex biological mechanisms which ensure relatively constant body weight over long time periods (Kuriyan et al., 2007). In the current study, the increase in the body weight of all experimental groups was similar, suggesting that saffron extracts did not induce significant changes in the appetite of mice and did not exert any undesirable effects on their metabolic growth and general health status. In addition, the water and feed consumption among the groups from the acclimatization time till the end of the experiment followed a steady pattern without any alterations. It suggests the normal processing of protein, carbohydrates and lipids metabolism inside the body of the animals, because these nutrients perform a key role in maintaining different physiological functions of the body (Saleem et al., 2017). The evaluation of such parameters is important to ensure the safety of

products with therapeutic applications, as proper intake of water and nutrients are essential to the physiological state of the animals (de Oliveira et al., 2018).

The hematological parameters are considered to be the most sensitive markers of the physiological changes in response to any toxic substance (Saleem et al., 2017). Additionally, it helps in providing information about the hematopoietic system and immunological responses. For example, higher level of leukocytes in the body is usually considered as a marker of stress and represents an immune response (de Oliveira et al., 2018). Therefore, the evaluation of the hematological parameters is important in animals, as it can point directly to the systemic effects caused by the administered drug. From the results, none of the hematological parameters were significantly different among treated and control groups in both genders. However, a significant increase in platelet count ($616.6x10^3/\mu L$) of SEE treated male group was observed compared to the control group ($548.4x10^3/\mu L$). Given the physiological function of platelets in blood coagulation, in some conditions an increase in platelets could show a toxicological effect by formation of thrombosis in blood vessel; while its reduction may cause adverse clinical implications, such as hemorrhage (Dongmo et al., 2019). However, the differences in platelet count in our experiment were not considered to be toxicologically meaningful.

Biochemical analysis of serum plays a major role in the assessment of the possible deleterious effects induced by the administered drug. Alteration of the level of three enzymes such as ALT, AST and ALP is normally used as a marker of hepatotoxicity (Lee et al., 2014). Among these enzymes, ALT is present in serum and numerous tissues, while AST is present in the liver, brain, kidneys, skeletal muscle, heart and red blood cells (You et al., 2007). When liver cells are disrupted by hepatotoxic substances, these two enzymes are released into the

blood stream (Dongmo et al., 2019). The third enzyme, ALP is found in the liver, kidneys, bone, small intestine, other body organs and placenta in pregnancy. ALP is considered as the standard marker of biliary tract diseases (Saleem et al., 2017). The level of ALP can be used to diagnose liver disease or bone disorders.

Relatively to hepatic functions, the results from the biochemical analysis revealed that all the blood parameters (ALT, AST, and ALP) remained unchanged. Nonetheless, the amounts of all biochemical parameters were slightly higher in PEE treated female mice as compared with the control group. Based on the serum levels of these enzyme indicators, saffron extracts did not show any toxic effects on liver tissue of mice. Likewise, oral administration of saffron ethanolic extracts showed no significant alteration on serum level of lipid-related markers including total cholesterol and triglycerides in male and female mice, when compared to their respective controls. Hence, these findings suggest that saffron at the dose level of 2000 mg/kg body weight has no deleterious effect on lipid metabolism, since no significant changes were observed in cholesterol levels of saffron administered mice. Similar results with single oral administration of 2000 mg/kg were observed by Halim et al. (2011) in *Carica papaya*, Ekanayake et al. (2019) in *Cocos nucifera* and Pariyani et al. (2015) in *Orthosiphon stamineus*, using the same toxicological method study.

Conventionally, organ weight changes have long been accepted as a sensitive indicator of toxicity to evaluate physiologic perturbations and target organ injury (Michael et al., 2007). A decrease in organ weight suggests necrosis while an increase indicates the occurrence of hypertrophy in the target organ (Ugwah-Oguejiofor et al., 2019). Age, sex, animal strains and environmental and experimental conditions are some of the key factors that may influence organs weight in animals (Piao et al., 2013). Organ weight background data is not

only important to evaluate whether or not the animal organ weights of the treatment groups are in the range of background data but also provide an important reference to pathologists for gross anatomy and microscopic examinations (Okamura et al., 2011).

During 14 days of treatment, no significant variation in ROW was observed between the treatment and control groups demonstrating the harmless nature of saffron ethanolic extracts. SEE treated mice of either sex showed slightly higher ROW of liver, pancreas and heart compared to the control. This increase could be considered incidental with no toxicological significance since the difference is not statistically significant. On the contrary, the ROW of liver and kidney in PEE treated mice of both sexes was slightly lower than control. Moreover, no evidence of gross abnormality, organ swelling, atrophy, or hypertrophy in the organs was observed at necropsy showing that saffron ethanolic extracts at 2000 mg/kg may not induce any toxic alterations on the organ weights of mice. However, the lack of deleterious effects on the studied organs does not attest total absence of toxicity because the manifestations of toxicity occur at the molecular level before expanding to the whole tissues and organs (Dongmo et al., 2019).

The histopathological examination serves as a supportive evidence for biochemical and hematological analysis (Traesel et al., 2016). Histopathological sections of internal organs such as heart, maintained normal architecture of the cardiac myocytes with the absence of cardiac myopathy, myofiber degeneration, necrosis, vacuolation and mononuclear cell infiltration. Likewise, renal tissues of the saffron ethanolic mice exhibited normal architecture with no evidence of glomerulosclerosis, interstitial inflammation, or parenchymal scarring. Also, the photomicrographs of pancreatic tissue did not show any abnormalities confirming the safety of the extract in the pancreatic tissue. Similar results of

single oral administration at 2000 mg/kg with normal histology without any deleterious effect on histological features of the vital organs are also reported in other plants (Ekanayake et al., 2019; Halim et al., 2011; Pariyani et al., 2015). Based on the results observed, we suggest that saffron ethanolic extract was safe and could be well used for pharmacological and therapeutic purposes.

Hot plate is one of the oldest and frequently used animal models to quantify "pain-like" behaviors in rodents (González-Cano et al., 2020). Based on the species and strain of rodents used in clinical studies, nearly 12 different behaviors including grooming, freezing, sniffing, licking, stamping of the legs, leaning, and jumping have been measured in the hot plate assay (Deuis et al., 2017). In the present study, hot plate analgesic assay of saffron extracts demonstrated a time-dependent activity. Among all the extracts of saffron, SEE exhibited the highest analgesic value in delaying the mean paw licking time (13.50 ± 0.28 s) by suppressing nociception in paws. PEE also delayed the onset time of licking response (11.13 ± 0.35 s). However, CEE and LEE exhibited weak analgesic activity of 22% and 29% at 2 h, respectively.

Good analgesic drugs suppress the activity of nociceptors and exhibit least number of lickings in animals. As per findings, SEE and PEE thus were found as potent analgesic agents. Maximum analgesic activity of SEE might be due to the presence of carotenoids, such as crocetin, crocin, picrocrocin, and safranal, as carotenoids have been reported to suppress the synthesis of prostaglandin synthetase (Yang et al., 2013). This speculation is supported by another study, where 0.1 and 0.2 g/kg crocin showed significant anti-edematogenic potential in histamine-induced paw edema in rats (Tamaddonfard et al., 2012). However, according to Hosseinzadeh & Younesi (2002), aqueous and ethanolic stigma and petal extracts of saffron

at any dose exerted no significant analgesic effect in mice and suggested that the extracts might not act through central mechanisms, although drugs that alter the animals motor ability may enhance the licking duration on the hot plate method without acting on the central nervous system.

Inflammation serves as a body's defensive biological response to damaged cells and injured tissue. The existence of edema is amongst the major signs of inflammation (Gupta et al., 2015). Carrageenan-induced paw edema is an established method to investigate the anti-edematous activity of natural products in rodents (Masresha et al., 2012). In the present study, anti-inflammatory potential of saffron ethanolic extracts was studied after sub-planter injection of 1% λ-carrageenan into mice. Test samples represented significant anti-edematous potential by regulating biphasic acute inflammatory response induced by carrageenan and showed highest edema inhibition in mice after 4 hours.

Carrageenan induces inflammatory process in two phases. Initial phase, which occurs during the first 2.5 h post-carrageenan injection, is attributed to the release of mediators like serotonin, histamine, and bradykinin on vascular permeability. Serotonin and histamine are produced in the first 1.5 h, while bradykinin is produced within 2.5 h post-carrageenan injection. The final phase occurs from 2.5 to 6 h after carrageenan injection, is associated with the overproduction of prostaglandins in tissues (Masresha et al., 2012). In addition to these mediators, Nitric Oxide (NO) is also reported to play a key role in carrageenan-induced acute inflammation (Arulmozhi et al., 2005).

Among all the extracts, SEE effectively inhibited the increase in paw volume of carrageenan-induced edema showing maximum percentage of edema inhibition (77.33%) at the end of 4 h followed by PEE (70.50%) at 800 mg/kg dose. Diclofenac potassium used as

a standard drug reduced paw edema volume by 88.87%. Therefore, it can be assumed that the active constituents of the extract might inactivate initial and final phases of inflammation by suppressing the production and activity of chemical mediators of inflammation.

Phytochemicals screening of SAE, SEE, PAE, and PEE on acute inflammation by xylene-induced ear edema indicated that only SAE and SEE at higher dose possessed anti-inflammatory effects in mice. However, SAE, SEE and PEE showed significant activity against chronic inflammation using formalin-induced edema in rat paw (Hosseinzadeh & Younesi, 2002). In another study, saffron aqueous extract suppressed formalin-induced paw edema in the chronic inflammation but failed to show activity against acute phase of a formalin test (Arbabian et al., 2009). Similarly, intraperitoneal injection of stigma constituent, crocin at concentrations of 0.1 and 0.2 g/kg significantly attenuated paw thickness and infiltration of neutrophils in paw tissues (Tamaddonfard et al., 2012).

Kumar et al. (2012) examined various petal extracts of *C. sativus* Cashmerianus to assess anti-inflammatory effect by carrageenan-induced paw edema method. Among all the extracts, methanol extract (400 mg/ml) exhibited 63.16% inhibition of paw volume followed by aqueous (57.89%) and chloroform (50%) extracts. The phytochemical profile of *C. sativus* petal suggests that the anti-inflammatory properties of PEE might be due to the presence of flavonoid (kaempferol, 12.6% w/w) carotenoids (crocin, 0.6% w/w and crocetin) and anthocyanins.

Normal hemostatic process is meant to stop a cut or wound on blood vessels through platelet thrombus formation. Subsequently, there is an eventual elimination of the plug when healing is complete. It is a delicate multiphase mechanism that requires the interaction of platelets and the coagulation factors with blood vessels. A defect in any of these phases can

result in thrombosis or hemorrhage (Ogedegbe, 2002). Extracts of various herbal plants have been used for their haemostatic role in wound healing, anti-infective, and antineoplastic properties (Scarano et al., 2013). Ankaferd Blood Stopper, herbal extract from five plants (*Urtica dioica*, *Alpinia officinarum*, *Vitis vinifera, Thymus vulgaris,* and *Glycyrrhiza glabra*) is approved for the management of external hemorrhage and post-surgery dental bleedings in Turkey to attenuate blood clotting time effectively (Kurt et al., 2010). Also, crude extracts of *Fagonia cretica* (74.6%), *Hedera nepalensis* (73.8%), and *Phytolacca latbenia* (67.3%) revealed promising anticoagulant effect by delaying blood clotting time to 86.9 s, 84.3 s, and 67.5 s, respectively (Ismail et al., 2017).

In this study, saline and aspirin show a clear line of difference between the coagulation and anticoagulation by depicting blood clotting time of 38.33 s and 108.5 s, respectively. The most significant anticoagulant effect was reported by SEE with a coagulation time of 101.66 s followed by PCC (86.5 s), respectively. LEE showed blood clotting time of 66.83 s, representing moderate inhibitory effect on coagulation activity. CEE was least effective in anticoagulation property and showed an almost equal response to saline indicating neutral effect of the selected extract which can be used in the treatment of blood clotting disorders such as hemophilia. It is said that the corms of *C. sativus* Cartwrightianus have a protein factor involved in human platelet aggregation (Liakopoulou-Kyriakides et al., 1985). However, 5 years later it was reported that it contains both activator/inducer of platelet aggregation (Liakopoulou-Kyriakides & Skubas, 1990).

Crocin delayed blood clotting time and mitigated respiratory distress as a result of pulmonary thrombosis in mice, inhibited thrombosis in rats, and suppressed platelet aggregation in rabbits (Shiping et al., 1999). Crocetin significantly reduced collagen- and

ADP-induced platelet aggregation but failed to reduce arachidonic acid-induced platelet aggregation (Yang & Qian, 2007). Besides that, crocetin significantly reduced dense granule secretion, while neither platelets adhesion to collagen nor cyclic AMP level was affected by crocetin (Yang et al., 2008). Saffron stigma tablets (200 and 400 mg/day) assessed for short-term safety and tolerability in a limited number of volunteers showed that only 200 mg of saffron tablets reduced International Normalize Ratio, platelets, and coagulation time (Modaghegh et al., 2008). Later, in a double-blind, placebo-controlled clinical study with a large sample size, saffron tablets (200 and 400 mg/day) administration failed to show any major effect on coagulant and anticoagulant system after one month. The authors suggested that the case reports of hemorrhagic complications might be due to high saffron dose, high period of consumption, or idiosyncrasy activities (Ayatollahi et al., 2014).

Depression is one of the most serious psychiatric disorders affecting approximately 4.7% of the global population and ranked as the eleventh most frequent cause of disease burden worldwide (Haroz et al., 2017). Most of the patients have many concerns about commencing synthetic antidepressants in their recommended doses due to the anticipated adverse reactions such as libido, constipation, dry mouth, and dizziness (Khawam et al., 2006). Hence, extracts of medicinal plants provide the most effective sources of novel drugs showing promising results with minimum side effects in the routine treatment of depression (Hausenblas et al., 2013; Wang et al., 2010).

The FST or Porsolt swim test, is the most frequently used test to screen antidepressants among all rodents with more reliability, sensitivity, and specificity (Petit-Demouliere et al., 2005). Immobility or the flouting response of rodents in FST is traditionally considered an indication of depression and anxiety (Kayani et al., 2016). During the FST, treatment of

antidepressant drugs attenuate immobility, prolong its onset and delay active escape behaviors of the animal (Ismail & Mirza, 2015).

In the present study, a prominent difference in immobility time of saline (141.16 s) and fluoxetine HCl (41.33 s) used as negative and positive control respectively was observed representing the reliability of the test. Furthermore, administration of saffron extracts showed a significant effect on reducing the immobility time in comparison to the saline-treated group. PEE represented itself as a potential antidepressant drug showing a notable diminution in immobility period (69.66 s) followed by SEE (76.66 s). Moreover, CEE (96.50 s) indicated moderate antidepressant effect, whereas LEE (106.83 s) exhibited mild activity. The potent antidepressant effect exhibited by petal can be strongly linked to the presence of natural flavonoid, kaempferol as it significantly attenuated immobility behaviors in rats and mice, and showed an almost similar response to fluoxetine (Hosseinzadeh et al., 2007). Similarly, in an 8-week double-blind randomized clinical study, dried saffron petal (15 mg bid) had similar antidepressant effects as fluoxetine (15 mg bid) in treating patients with mild-to-moderate depression and no significant differences in observed side effects (Basti et al., 2007).

In an investigation comparing the efficacy of saffron stigma and corms with fluoxetine against depression, petroleum ether and dichloromethane fractions of saffron stigma and corms significantly reduced the immobility time in the tail suspension and forced swimming test at all doses (150, 300, and 600 mg/kg) without altering the locomotor behavior of mice during the open-field test. The authors highlighted that antidepressant effect of stigma extracts could be due to crocin analogs, particularly crocin 1. However, HPLC analysis of corm extract revealed the absence of safranal, crocin, crocetin, or kaempferol compounds

assuming the presence of other bioactive compounds in saffron corms showing a potent antidepressant effect which need to be further explored (Wang et al., 2010).

There is a growing interest in the use of plant-based medicament in the prevention and treatment of diabetes mellitus. Over the last 2500 years, there have been very strong traditional systems of medicine, such as Ayurveda, Kampo, Chinese and Unani practiced in the eastern continent and have blossomed into orderly-regulated systems of medicine. It is estimated that nearly 80% of the population in developing countries depend on herbal medicines for their primary healthcare needs (Khan & Ahmad, 2019).

Plants are the major sources of different drugs, and more than 800 plants have shown antidiabetic activity (Bari et al., 2020). Despite the use of saffron as a local treatment of diabetes, no scientific investigations have been documented on *in vivo* grown saffron stigma and other parts. In the present study, ethanolic extracts of saffron corm, leaf, stigma, and petal were evaluated for anti-diabetic potential in animal model. In the first step of the study, mice were induced diabetic through STZ injection mimicking the picture of T2D, the most common cause to humans. STZ, a glucosamine–nitrosourea compound produced by *Streptomyces achromogenes*, causes hyperglycemia by selective destruction of β cells in the islets of Langerhans (Zhu, 2022). The diabetic mice were confirmed by the presence of high fasting blood glucose level, which is a characteristic feature of diabetes.

During oral glucose tolerance test, the fasting blood glucose level was significantly elevated in STZ-induced diabetic mice as compared to the normal control group after 0, 30, 60 and 120 min of saffron treatment. This is due to the lack of insulin in diabetic mice by destructing the β cells which leads to hyperglycemia. Treatment of STZ induced diabetic mice with SEE at a dose of 50 mg/kg b.w. displayed significant anti-hyperglycemic activity

at various time durations followed by PEE. CEE group after treatment for 2 hours showed moderate response, while LEE treatment displayed poor response. These results are compatible with the findings reported by other investigators using saffron aqueous extract at a dose rate of 100 mg/kg per day which declined blood glucose level in STZ-induced diabetic mice at various time duration (Jiang et al., 2018).

In the glucose loaded hyperglycemias model, ethanolic extracts of saffron resulted in lower serum glucose levels as compared to mice treated with STZ alone during the 21 days experiment. SEE and PEE at the dose rate of 50 mg/kg per day reduced blood glucose level in diabetic mice from 267 mg/dL to 118 mg/dL and 257 mg/dL to 129 mg/dL respectively, after 3 weeks of drug administration. Furthermore, CEE and LEE showed moderate effect by lowering the glucose level from 268 mg/dL to 160 mg/dL and 258 mg/dL to 183 mg/dL, respectively. Previous studies reported that saffron stigma has various compounds like α-krustyn, crocin, tricrocin, and safranal having antioxidants effects which play an important role in mitigating impaired insulin production and action in insulin resistance and prevent diabetes-related complications (Samarghandian et al., 2014).

Arasteh et al. (2010) reported that hydro-methanolic extract of saffron at 50 mg/kg IP significantly declined serum glucose level after 2 weeks in comparison to physiologic serum and normal group of male rats. Likewise, saffron aqueous extract at different doses behaved differently i.e., 20 mg/kg/day significantly decreased glucose in STZ diabetic rats at the 4^{th} week, 40 mg/kg/day at the 2^{nd} week and 80 mg/kg/day from the 1^{st} week of the treatment (Samarghandian et al., 2014). According to the authors, the hypoglycemic property of saffron extracts seems to be attributed by mechanisms such as stimulation of glucose uptake by peripheral tissues (Xi et al., 2007), insulin resistance reduction (Yang et al., 2003) and

inhibition of intestinal glucose absorption (Youn et al., 2004). We observed that our results were reported by other researchers in which saffron or its active constituents particularly crocetin, crocin and safranal showed antihyperglycemic effect in animal models (Altinoz et al., 2015; Elgazar et al., 2013; Kianbakht & Hajiaghaee, 2011; Motamedrad et al., 2019; Samarghandian et al., 2013).

Hyperlipidemia is associated with coronary artery disease in diabetic patients (El-Tantawy & Temraz, 2019). Furthermore, high blood glucose is correlated with a high risk of dyslipidemia. Hyperglycemia tends to elevate the level of LDL and TG while decline the level of HDL (Uddin Zim et al., 2021). The current study conducted on mice supported this theory by revealing that serum level of LDL, TC and TG was significantly higher while HDL level was significantly lower in diabetic control compared with normal control. This abnormally high level of LDL, TC and TG is primarily due to the low activity of lipolytic hormones in the fat depots due to the action of insulin. Insulin activates the enzyme lipoprotein lipase under normal circumstances, which hydrolysis TG. However, lipoprotein lipase is not activated in diabetic state due to deficiency of insulin that results in hypertriglyceridemia and hypercholesteremia (Elgazar et al., 2013).

In saffron treated groups, SEE and PEE administration to diabetic mice reverted the level of serum lipid markers towards the normal control mice, whereas CEE showed prominent effects in decreasing the LDL contents only. The hypolipidemic effects of saffron might be due to the increased plasma lipid uptake by the liver and adipose tissue or by decreased hepatic cholesterol genesis and fatty acid synthesis as indicated by Samarghandian et al. (2017). In the current study, LEE administration was least effective in terms of restoring the serum levels of lipid biomarkers in diabetic mice. Regarding the hypolipidemic effects of

saffron, Sheng et al. (2006b) reported that crocin has lipid-lowering properties and selectively inhibits the action of pancreatic lipases as a competitive inhibitor. According to Altinoz et al. (2015), administration of crocin at 20 mg/kg/day elevated lipid profile in diabetic rats and demonstrated that crocin might prevent diabetes induced cardiovascular complications by reduction of oxidative stress and dyslipidemia. In another experiment, He et al. (2005) demonstrated that crocin possess a potent hypocholesterolemic and hypotriglyceridemic effect in atherosclerotic quails, suggesting that saffron may be considered as one of the best supplements in the treatment of cardiovascular diseases.

In the present study, liver functions tests included serum concentrations of ALT, AST, and ALP. The activities of ALT and AST are cytosolic marker enzymes reflecting hepatocellular necrosis (Fouad et al., 2019). Therefore, the activities of ALT, AST and ALP in the circulation were determined as indicators to hepatic damage. It was found that diabetic control mice showed significantly higher ALT, AST, and ALP concentration in comparison with normal control mice. Among the saffron extracts, administration of SEE and PEE to diabetic mice restored the concentration of above altered parameters towards the normal control, whereas CEE showed moderate effect. According to Arkkila et al. (2001), higher serum AST and ALT level is a common sign of liver diseases and observed frequently among people with diabetes than in the general population. Generally, increased generations of free radicals due to oxidative stress develop adverse effects such as hepatology and nephropathy disorders in diabetes mellitus. Therefore, the oxidative stress is a common pathogenetic mechanism contributing to the initiation and progression of hepatic damage in a variety of liver disorders (Medina & Moreno-Otero, 2005).

Elgazar et al. (2013) reported that saffron aqueous extract significantly reduced the serum levels of ALT, AST and ALP as compared to the diabetic control group. Moreover, the reduction in serum levels of liver biomarkers were more prominent with increasing dose of saffron extract. Similarly, pretreatment of CCl4-treated rats with the saffron stigma main constituent, crocin significantly reduced the CCl4-induced elevation of ALT, AST and ALP levels and showed detectable protective properties against CCl4-induced hepatotoxicity (Bahashwan et al., 2015). In a double-blind placebo-controlled clinical trial, no significant changes in serum levels of ALT and AST were detected for liver function tests after consuming hydroethanolic extract of saffron in diabetic people showing the safety and nontoxic effects for saffron (Moravej Aleali et al., 2019). However, further studies in this field are required.

With regard to kidney function test, serum creatinine, BUN and UA levels of diabetic control group were significantly higher as compared to that of the normal control group. SEE followed by PEE depicted the lowest values of all kidney biomarkers showing no significant difference to the control group. Similarly, insignificant alterations in creatinine, BUN and UA contents were observed in CEE treated mice, whereas LEE showed marginal effect. These results were in accord with the findings of another study, where oral administration of saffron ethanolic extracts at 200, 400 and 600 mg/kg significantly reduced Cr, UA and BUN concentrations compared to the positive diabetic control (Elgazar et al., 2013).

Higher serum creatinine content is indicative of reduction in glomerular filtration rate, which is often associated with elevation in serum urea and uric acid (Okokon et al., 2019). In a study conducted by Milajerdi et al. (2017), consumption of hydroethanolic saffron extract resulted in significant reduction in serum levels of BUN and UA in the intervention

group. However, according to Kianbakht & Hajiaghaee (2011), stigma constituents, safranal and crocin did not change Cr levels in diabetic rats. Therefore, more studies are needed to further clarify the positive effect of saffron extracts, particularly stigma and petal on renal function.

Histopathological assessment of STZ induced diabetic mice showed acute distortion, inflammatory cell invasion and reduction of β cell mass in pancreatic tissue, whereas hyperammonemia, lobular inflammation, abnormal patterns with lymphocyte infiltration along with cytoplasmic degeneration in liver tissue. These results are in accordance with the previous studies (Gopal et al., 2014; Zangeneh et al., 2018; Zhang et al., 2018). However, treatment with SEE to diabetic mice expressively restored the histological changes to near normal with slight lymphocyte infiltration and liver regeneration. The antidiabetic activity of saffron stigma may be due to the presence of carotenoids, such as crocin, picrocrocin, safranal and crocetin and/or flavonoids such as kaempferol. It is reported that carotenoids and flavonoids, particularly kaempferol along with triterpenoids and phenolics constitute the active biological principles of most medicinal plants with the most promising hypoglycemic and antidiabetic effects (Mohammed & Tajuddeen, 2022; Tran et al., 2020).

These findings were in line with study done by Elgazar et al. (2013), where treatment of saffron ethanolic extracts to alloxan-induced diabetic rats at 600 mg/kg b.w depicted normal histological structure of pancreas. However, slight hypertrophy of islets of langerhans at 400 mg/kg b.w and vacuolation of acinar epithelial lining in pancreatic tissue at 200 mg/kg b.w was demonstrated. Similarly, i.p injection of saffron extract (40 mg/kg b.w/day) restored the pathological changes in liver tissue of STZ induced diabetic rats and no considerable fatty change was observed (Mohammad et al., 2011).

In another study, treatment of saffron aqueous extract at 100 mg/kg/day for 6 weeks to STZ diabetic mice repaired the histopathological alterations in pancreatic tissues to normal (Jiang et al., 2018). Overall, this study supports the antihyperglycemic, antihyperlipidemic and antioxidant potential effect of the ethanolic extracts of saffron because of the presence of bioactive compounds, such as carotenoids and flavonoids.

CONCLUSION

Saffron is derived from the elongated dried stigmas of saffron (*Crocus sativus* L.) flowers, an autumn-flowering herbaceous plant that is propagated solely through clonal corm multiplication. The rate of natural propagation of corms is very low; hence an alternative *in vitro* culture method like micropropagation is urgently needed.

In the present study, corms were surface sterilized with various sterilization protocols and cultured on medium fortified with several concentrations of different phytohormonal combinations for ensuring bud sprouting, multiple shoots proliferation and cormlet production. It was observed that 2-3 min treatment of corms with 70% ethyl alcohol and washing in 40% clorox and 0.2% $HgCl_2$ for 5 min each ensured clean aseptic viable cultures.

Maximum buds sprouting response followed by highest number of multiple shoot primordia from the base of sprouted buds were obtained from MS enriched with BAP (4 mg/l) in combination with IAA (1 mg/l). Further sub culturing of shoot primordia in BAP (6 mg/l) and IAA (0.5 mg/l) was superior for achieving multiple shoots proliferation. MS medium supplemented with GA_3 (4 mg/l) and 4% sucrose exhibited significantly higher cormlet number and cormlet weight. However, media containing 2% sucrose and GA_3 (2 mg/l) gave highest root number and root length per explant. These observations could be used to produce pathogen-free corms of saffron and other related species.

Different doses of γ-rays (0, 10, 20, 30, and 40 Gy) through the Gammacell 220 irradiator were assessed for their potential effect on morpho-agronomic characteristics of saffron. It was shown that corms survivability, days to sprouting, sprouting percentage, plant height, days to flowering, and number of flowers formed and bloomed negatively affected with

increasing doses of gamma radiation. However, 10 Gy treatment stimulatively effected on various vegetative attributes resulting in maximum leaf number (5.93), leaf length (14.78 cm), early flowering (85 days) with longest harvest period (8.82 days) and maximum weight of progeny corms (4.07 g).

It was determined that radiation dose of 20 Gy was the most optimal dose for the reproductive traits of saffron showing the highest flower fresh (172.90 mg), flower dry weight (36.37 mg), stigma fresh weight (38.84 mg), stigma dry weight (7.72 mg), stigma length (4.96 cm), and number of progeny corms (5.25). Also, one of the 20 Gy plants showed various changes in flower shape and colour in the form of four irregular dark petals, tetrafid stigma, and two stamens compared with six normal-sized petals, trifid stigma, and three stamens in control. Higher doses of 30 Gy and 40 Gy were critical and lethal, respectively showing deleterious effects in terms of survival, vegetative as well as floral growth of saffron. Since, flower is the most valuable part of saffron responsible for imparting aroma, colour, and taste, low doses of gamma irradiation is the best alternative to create high-yielding and high-quality saffron cultivars.

According to the results, except planting depth, all other factors had a significant effect on floral and daughter corm related attributes. The highest growth yield for all variables was attained when bigger mother corms of 15 g were grown in soil containing cattle manure at 18 ºC. Also, saffron growth indices were significantly affected by planting seasons. Except for DCW, other growth indices after the end of 1st year were significantly higher than 2nd year experiment.

In the acute toxicity study, a single dose of orally administered saffron ethanolic extracts (corm, leaf, petal, and stigma) at 2000 mg/kg body weight of male and female mice was

monitored for 14 days. No mice in either gender during the acute toxicity study exhibited mortality or clinical signs of toxicity. Further, these mice did not show any significant change in their mean body weight, water and food intake, hematological, biochemical parameters, relative organ weight as well as in the results of their histopathological examinations compared to those of control group. According to results, the LD_{50} of all saffron ethanolic extracts is greater than 2000 mg/kg body weight.

In an attempt to explore the potential nutraceutical efficacy of corm, leaf, petal, and stigma of saffron ethanolic extracts, hot plate, carrageenan-induced paw edema, capillary tube and forced swim test were performed in mice for their analgesic, anti-inflammatory, anticoagulant, and antidepressant activity, respectively. The results indicated that among all the extracts, SEE represented maximum latency activity and edema inhibition followed by PEE. CEE and LEE displayed mild analgesic activity. Also, LEE and CEE exhibited mild to moderate response against inflammation.

The coagulation time of SEE was almost equivalent to the standard drug, aspirin suggesting a strong anticoagulant effect followed by PEE. LEE represented moderate inhibitory effect on coagulation activity, while CEE showed neutral effect. Additionally, PEE and SEE also expressed itself as potential antidepressants, while CEE and LEE indicated moderate to mild antidepressant efficacy.

During oral glucose tolerance test, the fasting blood glucose level was significantly elevated in STZ-induced diabetic mice as compared to the normal control group after 0, 30, 60 and 120 min of saffron treatment. Treatment of diabetic mice with SEE and PEE showed a significant decrease ($p<0.05$) in blood glucose level at various time durations. CEE and LEE group after treatment showed moderate and poor response.

Regarding hypoglycemic and hypolipidemic effect of saffron extracts, SEE and PEE administration to diabetic mice reverted the level of glucose and serum lipid markers such as LDL, HDL, TC and TG towards the normal control mice, whereas CEE showed prominent effects in decreasing the LDL contents only and LEE administration was least effective.

During the renal and liver function tests, administration of SEE and PEE to diabetic mice restored the concentration of kidney biomarkers such as Cr, UA and BUN and liver biomarkers such as ALT, AST, and ALP towards the normal control, whereas CEE showed mild response followed by LEE.

Histopathological assessment of STZ induced diabetic mice showed acute distortion, inflammatory cell invasion and reduction of β cell mass in pancreatic tissue, whereas hyperammonemia, lobular inflammation, abnormal patterns with lymphocyte infiltration along with cytoplasmic degeneration in liver tissue. However, treatment with SEE to diabetic mice expressively restored the histological changes to near normal with slight lymphocyte infiltration and liver regeneration.

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