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IN VITRO ANTIOXIDANT POTENTIAL OF VARIOUS EXTRACTS OF *MECONOPSIS ACULEATA*

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ABSTARCT

Meconopsis aculeata is used in Kashmir as a traditional medicine against various ailments. The current study was designed to investigate the in vitro antioxidant potential of different extracts of Meconopsis aculeata against DPPH radical scavenging activity, hydroxyl radical scavenging activity, superoxide radical scavenging activity, reducing power and inhibition of microsomal lipid peroxidation was also calculated. Phytochemical screening of the plant extracts revealed the presence of most of the phytochemicals in methanol and ethanol extracts. Total phenolic content of the extracts were also evaluated during our study. Highest phenolic content of 540 mg GAE/g was found in methanol extract followed by ethanol extract (478 mg GAE/g) and ethyl acetate extract (296 mg GAE/g). DPPH radical scavenging of methanol extract was maximum (90.89%) at the concentration of 700 µg/ml. Ethanol and ethyl acetate extracts showed percent inhibition of 85.67% and 56.18% at the same concentrations respectively. We observe an increase in reducing power of the extracts with increase in concentration. Maximum inhibition of superoxide radical (80%), hydroxyl radical (80%) and lipid peroxidations (84.78%) were observed at the highest concentration of methanol extract used. The results obtained indicate that Meconopsis aculeata possesses free radical scavenging activity and can act as a potent source of antioxidants against oxidative stress related diseases.

Key words: DPPH, lipid peroxidation, Meconopsis aculeata, radical scavenging, reducing power.

I. INTRODUCTION

Reactive oxygen species (ROS) are generated by living system during cellular metabolism or by exogenous sources. These species are required for normal cellular processes but at higher concentrations these reactive species cause deleterious effects to the cells [1]. ROS are highly reactive species due to the presence of unpaired electrons and tend to attack different biological molecules to get stabilized and in this process they initiate a cascade of reactions generating more and more free radicals [2]. The major proportion of ROS in our body is produced by the mitochondrial electron transport chain. Other endogenous sources of ROS are Cytochrome P-450, oxidative enzymes like xanthine oxidase and immune cells like macrophages and neutrophils [3]. The exogenous sources of ROS include tobacco smoke [4], radiation, UV light [5] and heavy metal ions [6]. Oxidative stress has been found to contribute to various pathological conditions like cancer, diabetes, asthma,

pulmonary diseases and hypertension [7]. These free radicals have special affinity for lipids, proteins and DNA [8]. The integrated enzymatic and non-enzymatic antioxidant system combats against this oxidative stress. During the pathological state the overwhelmed antioxidant system needs exogenous supply of antioxidants to fight the oxidative stress. Dietary antioxidants are able to prevent cells against oxidative stress [9]. The synthetic antioxidants available are associated with potent toxicity which leads to the shift in the area of treatment of oxidative stress from synthetic to natural therapy [10]. The aim of this study was to look for a natural antioxidant as an alternative to synthetic antioxidant in different extracts of *Meconopsis aculeata*. The plant under study is being used in folklore medicine for treatment of ulcers, pharyngitis and various inflammatory disorders.

II. MATERIALS AND METHODS:

2.1. Collection of Botanic material:

Whole plant of *Meconopsis aculeata* was collected from high altitudes of Margan pass, Jammu and Kashmir during the month of August. Botanical identification of the collected material was done by the Centre of Plant Taxonomy, Department of Botany, University of Kashmir under the reference number 2690-KASH.

2.2. Preparation of extracts:

The collected plant material was cleaned and shade dried at room temperature. The completely dried material was ground in powder using a grinder. The powder was extracted with different solvents using soxhlet extractor, filtered and concentrated with rotary evaporator. The temperature of soxhlet was set according to the boiling temperature of respective solvents. The residual solvents were evaporated in a hot water bath kept at 40ºC. The solvent free extracts were then stored at 4ºC till further use.

2.3. Phytochemical screening:

Qualitative analysis of different phytoconstituents in *Meconopsis acuelata* whole plant extracts was done by various reported methods.

2.4. DPPH radical scavenging assay:

DPPH radical scavenging assay was conducted according to the method described by Braca *et al*. [11] with some modifications. Different concentrations of plant extracts ranging from 100µg/ml to 700µg/ml were incubated with 10mg% methanol solution of DPPH for 30 minutes in dark. The absorbance of the samples was measured at 517 nm. BHT was used as standard. The percentage inhibition of free radical was calculated according to the following formula:

$$\% \text{ inhibition} = \frac{Ac - As}{Ac} \times 100$$

Where A_c is the absorbance of control i.e. without sample and A_s is the absorbance of sample.

2.5. Total phenolic content:

The quantification of total phenols in different extracts of *Meconopsis aculeata* was determined according to the method of Singleton *et al.* [12]. The yellow color of Folin-Ciocalteu reagent changes to blue when it detects phenolic compounds. Different plant extract concentrations were incubated with Follin's reagent in presence of alkaline conditions followed by incubation of reaction mixtures at room temperature for 20 minutes. The absorbance was read at 765nm. The content of phenols in the extracts was estimated using the standard curve of gallic acid and the results were expressed in milligrams (mg) gallic acid equivalent (GAE) per gram of plant extract.

2.6. Reducing power assay:

The reducing power of plant extracts was determined according to the method of Oyaizu [13]. In this method, the potential reducing agents convert potassium ferricyanide (Fe^{3+}) to potassium ferrocyanide (Fe^{2+}) which then reacts with ferric chloride to form blue colored ferric ferrous complex. Different concentrations of the extracts were incubated with 0.2 M phosphate buffer (pH 6.6) and 1% potassium ferricyanide. The mixture was incubated at 50ºC for 20 min followed by addition of 10% trichloroacetic acid. The reaction mixture was centrifuged at 3000 rpm for 10 min. The supernatant was then added by equal amount of distilled water and 0.5ml of ferric chloride. The absorbance was read at 700 nm. BHT was used as standard.

2.7. Superoxide radical scavenging assay:

The superoxide radical scavenging assay was performed by riboflavin/light/NBT method. The assay was based on the scavenging capability of superoxide ion generated by auto oxidation of riboflavin by light which can reduce NBT to blue colored formazon [14]. Different concentrations of plant extracts were incubated with 50mM phosphate buffer (pH7.6), 0.12mM riboflavin, 12mM EDTA, and 1.5mM NBT. The reaction mixture was illuminated for a brief time of 90 sec. The absorbance was measured at 590nm. BHT was used as positive control.

The percentage inhibition of superoxide anion by the extracts was calculated by using the following formula:

$$\% \text{ Inhibition} = 1 - \frac{As}{Ac} \times 100$$

2.8. Hydroxyl radical scavenging assay:

The hydroxyl radical scavenging activity of plant extracts was performed according to the method of Halliwell *et al*. [15]. The thiobarbituric acid reactive species are generated from the degradation of deoxyribose sugar when exposed to hydroxyl radical which is in turn generated by Fentons reaction ($Fe^{3+/}$ascorbate/EDTA/H_2O_2 system). Different concentrations of plant extracts were incubated with the reaction mixture containing 25mM deoxyribose, 20mM ferric nitrate, 10mM ascorbic acid and 0.2M H_2O_2 . The reaction mixture was incubated at 37ºC for 30 minutes followed by addition of 1ml 10% TCA and then centrifuged at 5000rpm for 5 minutes. 1ml

of 1% thiobarbituric was added and the mixture was heated at 100ºC till color appeared in control tube. The color intensity was spectrophotometrically measured at 532 nm and the percent inhibition of hydroxyl radical was calculated as follows:

$$\% \text{ inhibition } = \frac{Ac - As}{Ac} \times 100$$

Where A_c is the absorbance of control and A_s is the absorbance with extract.

2.9. Lipid peroxidation inhibition assay:

2.9.1. Liver microsomes preparation:

Liver from freshly killed rat was excised and immediately transferred into ice cold 0.9% NaCl. The tissue was properly washed to remove all the blood. Any kind of tissue debris or extraneous material was properly removed. The tissue was blotted between the folds of a filter paper and weighed. 10% (w/v) homogenate was prepared in homogenizing buffer (50mM Tris HCl, 1.15% KCl, pH 7.4) with teflon homogenizer. The homogenate was filtered through a muslin cloth and centrifuged at 5000 rpm for 10 minutes at 4°C to separate nuclear debris. The supernatant was again centrifuged at 15000 rpm for 20 minutes at 4°C. The supernatant obtained at this step is post mitochondrial supernatant and if required, can be stored at 4°C till further use. The PMS was again centrifuged at 60000 rpm for 2 hours to acquire microsomal pellet which was then resuspended in a minimum volume of 0.25 M sucrose and stored at -80°C for further use.

Liver microsomal peroxidation was carried out by method described by Urata *et al*. [16]. Different concentrations of extracts were incubated with liver microsomes. 20mM ferric nitrate and 100mM ascorbic acid was added to induce lipid peroxidation in microsomes. The reaction mixture was incubated for an hour at 37ºC. Termination of reaction was done by adding 10% TCA and later the reaction mixture was boiled for 15 minutes after adding 1% TBA. The samples were read at 532 nm. Percentage inhibition was calculated using the below formula:

$$\% \text{ Inhibition } = 1 - \frac{As}{Ac} \times 100$$

Where A_c is the absorbance of control and A_s is the absorbance with extract.

2.10. Statistical analysis:

The result values of the experiments are expressed as mean ± standard deviation. All the experiments were carried out at least thrice. Evaluation of the results was done using SPSS (version 12.0) and Origin 8.1 version software.

III.RESULTS

3.1Phytochemical screening of *Meconopsis aculeata* extracts

The phytochemical analysis of different extracts of *Meconopsis aculeata* revealed the presence of maximum phyto constituents in polar solvent extracts. The phytochemical analysis of different extracts is shown below in Table 1. The results revealed the presence of maximum content of flavonoids in methanol and ethanol extracts followed by ethyl acetate and aqueous extracts. Maximum content of Phenols was present in methanol extract followed by ethyl acetate and ethanol extracts whereas aqueous and hexane extract showed less amounts of phenols. Aqueous extract contained the maximum amounts of saponins followed by methanol extract whereas rest of the extracts did not contain saponins. Hexane extract showed the presence of maximum steroids than methanol, ethyl acetate and ethanol extracts whereas aqueous extracts was found to be devoid of steroids. Terpenoids were found to be present in maximum amounts in methanol extract and minimum in ethyl acetate extract.

Table 1: Phytochemical analysis of different extracts of *Meconopsis aculeata*

Phytochemicals	Hexane extract	Ethyl acetate extract	Methanol extract	Ethanol extract	Aqueous extract
Flavonoids	--	++	+++	+++	+
Phenols	+	++	+++	++	+
Saponins	-	-	+	-	+
Steroids	++	+	+	+	-
Terpenoids	-	+	+++	++	-

Table 2: IC50 values (µg/ml) of different extracts of *Meconopsis aculeata* with reference to standard BHT

Extracts	DPPH Radical	Superoxide radical	Hyrdoxyl radical	Lipid peroxidation
Methanol	237	42.09	345	33.5
Ethanol	290	52.75	423	39.5
Ethyl acetate	560	62	665	56.5

3.2 DPPH radical scavenging activity of *Meconopsis aculeata*

The methanol, ethanol and ethyl acetate extracts of *Meconopsis aculeata* showed dose dependent scavenging of DPPH free radical. The maximum DPPH scavenging activity was observed in methanol extract (90.89±0.543) followed by ethanol (85.67±0.42) and then ethyl acetate extract (56.18±1.21) at highest concentration of 700µg/ml as shown in Fig.1. The IC50 values of methanol, ethanol and ethyl acetate extracts were found to be 237, 290 and 560 µg/ml respectively (Table 2).

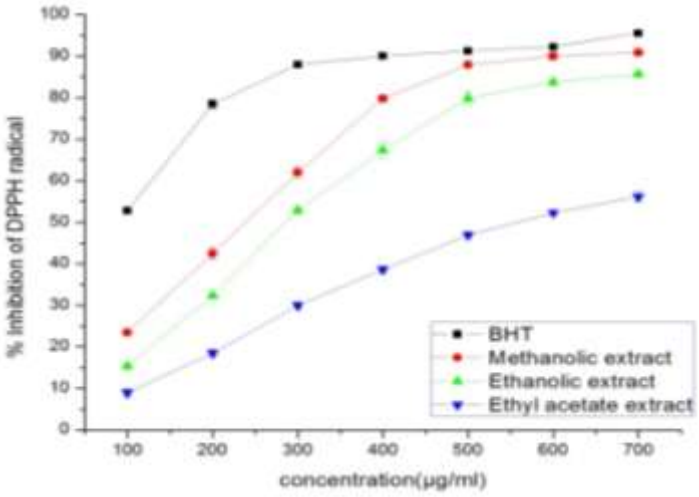

Figure 1: percent inhibition of DPPH free radical by different extracts of *Meconopsis aculeata*

3.3 Total phenolic content of *Meconopsis aculeata* extracts

The total phenolic content analysis of different extracts of *Meconopsis aculeata* followed a decreasing trend in order of methanol > ethanol > ethyl acetate extract (Fig.2). The highest phenolic content of 540mg GAE/g extract was found in methanol extract whereas ethanol and ethyl acetate extracts were found to contain 478 and 296 mg GAE/g extract phenols respectively.

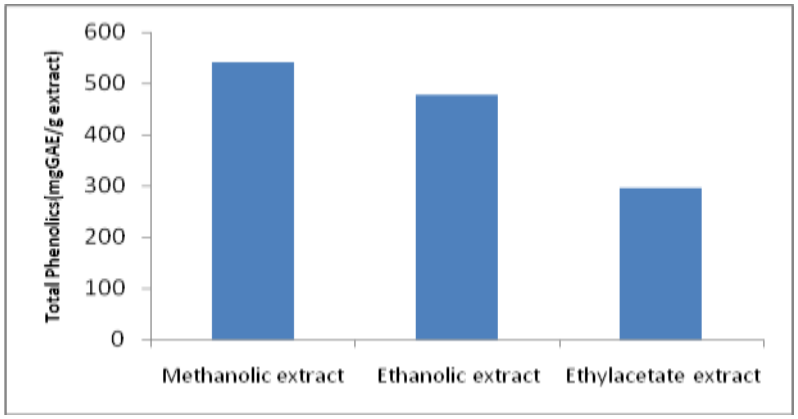

Figure 2: Total phenolic content of different extracts of *Meconopsis aculeata*

3.4 Reducing power of *Meconopsis aculeata* extracts

The reducing power of different extracts of *Meconopsis aculeata* followed a concentration dependent manner as shown in Fig.3. Higher the value of absorbance, strong is the reducing activity of the sample. The absorbance at 700nm increased with 100-700 µg/ml of extract from 0.081 to 0.527 for methanol extract, 0.054 to 0.39 for ethanol extract and 0.039 to 0.282 for ethyl acetate extract.

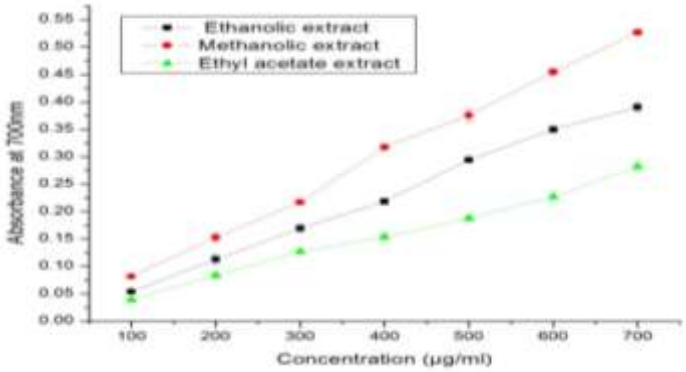

Figure 3: Reducing power of different extracts of *Meconopsis aculeata* represented by increase in absorbance with increase in concentration

3.5 Superoxide radical scavenging activity of *Meconopsis aculeata*

Methanol and ethanol extracts of *Meconopsis aculeata* were found to scavenge the superoxide radicals much efficiently than the ethyl acetate extract. The scavenging of free radical was concentration dependent in all the extracts while methanol extract showed the maximum percent inhibition as compared to other extracts at all the concentrations. The highest concentration of methanol, ethanol and ethyl acetate extracts showed 80.015±1.03, 69.78±0.98 and 55.24±0.95 percent inhibition of superoxide radical respectively as shown in Fig.4. The IC50 values of the plant extracts for superoxide scavenging activity were 42.09, 52.75 and 62µg/ml respectively (Table 2).

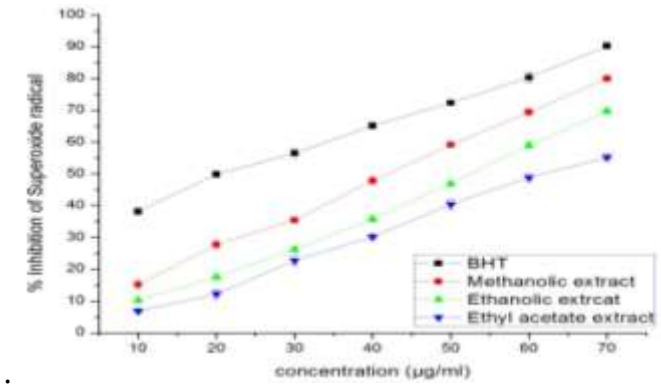

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Figure 4: Superoxide radical scavenging property of different extracts of *Meconopsis aculeata*

3.6 Hydroxyl radical scavenging activity of *Meconopsis aculeata*:

Meconopsis aculeata extracts showed significant stabilization of hydroxyl radical. The extracts exhibited the radical scavenging activity in a concentration dependent manner with maximum inhibition shown by methanol extract (80.01±1.03) (Fig. 5). Ethanol and ethyl acetate extracts showed maximum percent inhibition of 69.78±0.98 and 55.24±0.95 respectively. The methanol, ethanol and ethyl acetate extracts showed IC50 values of 345, 423 and 665 µg/ml respectively (Table 2). BHT was used as standard in this assay.

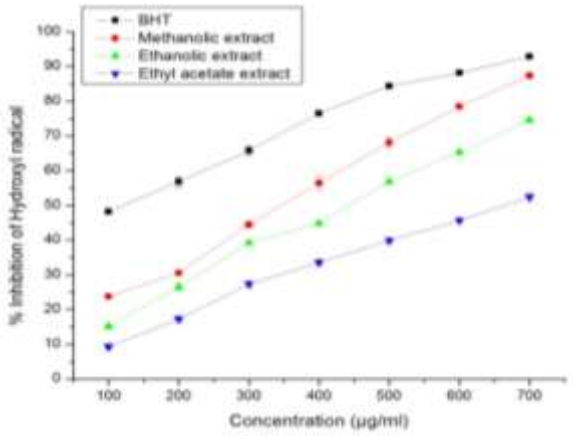

Figure 5: Percent inhibition of hydroxyl radical by different extracts of *Meconopsis aculeata*

3.6 Lipid peroxidation inhibition assay

The production of TBARS was significantly inhibited by *Meconopsis aculeata* extracts in a concentration dependent pattern. The methanol extract at higher concentration of 70 µg/ml showed 84.78±1.05 percent inhibition of TBARS production. Ethanol and ethyl acetate extracts showed 74.33±1.14 and 58.93±0.38 percent inhibition of TBARS respectively (Fig. 6). The IC50 values of the extracts were 33.5 µg/ml (methanol), 39.5 µg/ml (ethanol) and 56.5 µg/ml with ethyl acetate extract (Table 2).

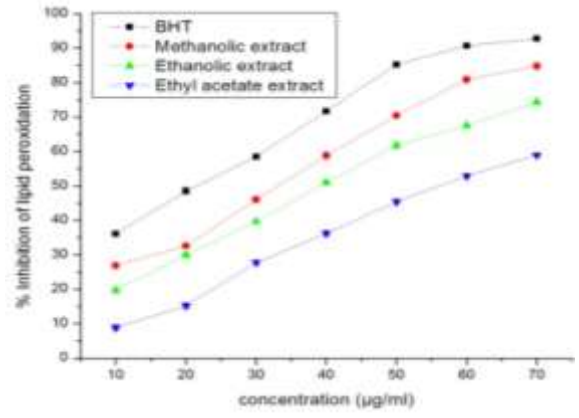

Figure 6: Percent inhibition of lipid peroxidation by different extracts of *Meconopsis aculeata*

IV.DISCUSSION

Oxidative stress damages the cellular components like proteins, DNA and membrane lipids which may cause cell death [17]. It has also been found to be a major cause of various diseases like cardiovascular diseases, diabetes, degenerative diseases, inflammation, etc. [18]. The destructive impact of oxidative stress on human health has become an important issue [19]. The endogenous anti-oxidant system is not able to handle the load of reactive species during oxidative stress indicating the need of dietary antioxidant consumption. According to the World Health Organization (WHO) almost 80% of the world population utilizes the traditional herbal medicines, either the herbal extracts or their active constituents. Studies have suggested the direct relationship between the dietary consumption of antioxidants rich food and sound health [19]. The side effects, lack of availability and high cost of synthetic anti-oxidants make their use challenging against oxidative stress [20]. However, the abundance of plant based natural antioxidants which are free from side effects and are less expensive can be considered for alternate therapy against oxidative stress.

Meconopsis aculeata is a perennial herb belonging to family Papaveraceae. Whole plant of *Meconopsis aculeata* is used as blood purifier and to reduce blood pressure. In Tibetan medicine it is used as analgesic and febrifuge and to treat inflammation from broken bones. The experiments carried out on different extracts of this plant revealed its potency to scavenge free radicals like superoxide, hydroxyl radical etc. which are generated during oxidative stress conditions. The methanol extract of this plant was found to be more potent in scavenging most of the free radicals when compared to other extracts.

DPPH radical scavenging assay is one of the most reliable and quick methods for determining the radical scavenging property of antioxidant as it is accepted as model free radical originating from lipids [21]. The stable purple colored DPPH free radical was scavenged by antioxidants present in the extracts changing the color of solution from purple to yellow. More the antioxidant potential of the extract less is the intensity of purple color in the solution. In our experiments methanol extract of *Meconopsis aculeata* was more potent in neutralizing the DPPH free radical followed by ethanol and ethyl acetate extracts. Similarly methanol extract of *Euphorbia wallichii* has shown the best DPPH scavenging activity from all the solvent extracts [22].

Phenolic compounds found in herbs are able to scavenge free radicals thereby inhibiting lipid peroxidation [23]. The total phenolic content of different extracts of *Meconopsis aculeata* was assayed by Folin-ciocalteu method. Methanol extract was found to possess maximum phenolic compounds followed by ethanol and ethyl acetate extracts. The reduction of Fe^{3+} to Fe^{2+} is carried out by potent reducing agents which lead to the development of bluish color in the solution. High reducing power is indicated by high absorbance at 700nm. Methanol extract of *Meconopsis aculeata* was found to be more potent reducing agent when compared to ethanol and ethyl acetate extracts. Irshad *et al*., while studying the antioxidant activity of *Cassia fistula* extracts, found similar results [24].

Superoxide anion free radical is involved in initiating redox reactions related to aging [25]. It also acts as an initiator for production of other free radicals like hydroxyl radical and hydrogen peroxide radical which are responsible for causing damage to DNA and proteins [26]. All the extracts of *Meconopsis aculeata* were able to

scavenge superoxide radical. Methanol extract was again more potent than ethanol and ethyl acetate extracts. The decrease in the absorbance with increasing concentration of extract was due to the less formation of formazon which is produced by the reduction of NBT by superoxide radical. It implies that the superoxide radicals were being scavenged by antioxidant agents in these extracts thereby not providing enough free radicals for the reduction of NBT. The scavenging potential of the extracts was found to be concentration dependent.

Hydroxyl radicals cause damage to lipids, proteins and DNA [27]. Hydroxyl radical is generated by Fenton's reaction which on reacting with TBA produces TBA reactive species, predominantly malondialdehyde. The amount of Malondialdehyde produced is directly proportional to the pink color intensity which is measured at 532nm. Methanol extract of *Meconopsis aculeata* was able to scavenge the hydroxyl radical more effectively than methanol and ethyl acetate extracts. *Podophyllum hexandrum* extracts showed similar results when assayed for hydroxyl radical scavenging activity [28].

The peroxidation of membrane lipids is a considered as a hallmark of oxidative damage [29]. It leads to membrane protein cross linking, alteration in membrane fluidity, and formation of lipid-protein and lipid-DNA adducts which definitely alters the cellular functions [26]. Lipid peroxidation has been considered among the causes of cancer [30] and cardiovascular diseases [1]. Phytochemicals like flavonoids attenuate the lipid peroxidation process by stabilizing the peroxyl radical [31]. The initiation of lipid peroxidation in rat liver microsomes by Fe^{2+}/ascorbic system is well studied [32]. High content of flavonoids and phenols in the methanol extract of *Meconopsis aculeata* might be a contributing factor towards its high inhibition of TBARS formation as compared to the ethanol and ethyl acetate extract. *Abrus precatorius* leaf extracts have also showed similar results for lipid peroxidation assay [33].

V.CONCLUSION

The present study provided evidences in support of *Meconopsis aculeata* possessing potent free radical scavenging property. The methanol extract of this plant is more promising in its antioxidant property which may be mainly due to the presence of high phenolic content. The methanol extract can be further explored for the isolation of its active constituents.

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