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Phytochemical investigation and pharmacological evaluation of methanolic flower extract of *Saussurea heteromalla*

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Abstract: *Saussurea heteromalla* is the one of specie of *Saussurea* plant belonging to family *Asteraceae*. The *Saussurea heteromalla* found extensively in Pakistan. The literature review highlights numerous biological aspects of *Saussurea heteromalla*. This research therefore aims to assess its potential anti-inflammatory, antioxidant, anti-cancer. Carrageenan induced rat paw edema model was used for the evaluation of anti-inflammatory activity. DPPH method was used to evaluate anti-oxidant activity. The MTT (3- [4, 5-dimethylthiazol-2-yl]-2, 5 diphenyl tetrazolium bromide) test was used to assess the viability of the cells for the assessment of the cytotoxic effect of the extract. Methanolic *Saussurea heteromalla* flowers extract analysis carried by GC-MS, result 19 different peaks. The methanolic extract of *Saussurea heteromalla* at 400mg/kg dose have equal anti-inflammatory action when compare with standard that is diclofenac sodium. Anti-oxidant activity of methanolic extract is also very good. IC50 value of methanol extract was 25µg/ml. 18.72% cell survive out of 100 when methanolic flower extract of *Saussurea heteromalla* was given at the dose of 400mg, which shows the cytotoxic effect. This activity shows that plant *Saussurea heteromalla* methanolic flowers extract have anti-inflammatory, anti-oxidant, cytotoxic effect. The isolation and characterization-based investigations proclaiming the biologically leading active molecule are worthy for further study in this regard.

Keywords: MTT, (3-[4, 5-dimethylthiazol-2-yl]-2, 5 diphenyl tetrazolium bromide), GC-MS, gas chromatography mass spectrometry, UV, ultraviolet, DMSO, dimethylsulphoxide, ROS, reactive oxygen species.

INTROUDUCTION

Different types of organic compounds are available in plants such as alkaloids, tannins, carbohydrates, steroids etc. which are widely used for the treatment purpose (Yadav and Agarwala, 2011). Different forms of diseases are cured historically with medicinal plants or in the form of a pure active principle (KumarVan and Staden, 2016). Owing to the side effects of prescription medications, failure of modern therapies against chronic diseases and microbial resistance due to which a major resurgence of herbal product use has occurred in recent years. Ethno-medicinal science plays a crucial role in identify new medicines from green pharmaceuticals and indigenous medicinal plants are getting popularity and exceptional significance due to vast opportunities (Tomlinson and Akerele, 2015). Nearly 75% of the plant based medicinal entities used worldwide believed to have originated from traditional medicine. It has been stated that over 60% of the cancer medication on the market or in research is focused on natural products. Around 80% of antimicrobial, cardiovascular and anticancer drugs are currently extracted from plant sources. 70% of the licensed anti-cancer drugs are based on natural ingredients (Sen and Chakraborty, 2017). Our immune system responds and results in inflammation when pathogens such as viruses and bacteria invade the body, but if excess inflammatory mediators such as prostaglandins and nitric oxide have been released, they may lead to a variety of diseases such as septic shock, arthritis and cancer (Delves and Roitt, 2000). Synthetic agents have serious side effects, including stomach ulcer and bleeding, so we tried to substitute synthetic agents with other products that were obtained with good efficacy and low toxicity from medicinal plants (Poggi *et al.*, 2000) The discovery of new compounds with enhanced pharmacological activity may also contribute to these compounds. (Khuda *et al.*, 2014) When chemical bonds of molecules breakdown it results into free radical having one electron. Free radicals are often generated by collusion between non-radical species. (Cheeseman and Slater, 1993) Free radicles of reactive oxygen contribute to a variety of diseases, including congestive heart failure, ischemic heart disease, high blood pressure, arterial blockage etc. (Freeman and Crapo, 1982) Pollutants, smoking and UV rays may be the cause of free radical development. Antioxidants are agents which inhibit molecular oxidation and thus prevent the development of free radicals. (Kaur and Kapoor, 2002) Vitamin C, vitamin A and phenolic compounds are examples of antioxidants. (Bahorun *et al.*, 2006) Skin is the largest body organ that protects our body from multiple

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infections and other hazardous environmental factors. (Adams and Adams, 1983) UV radiation is the predominant cause of skin cancer. The skin cancer also includes genetic and environmental causes (Delves and Roitt, 2000) Immunosuppressed patients are more vulnerable to skin cancer (Nichols and Katiyar, 2010). *Saussurea heteromalla* is the one of specie of *Saussurea* plant belonging to family Asteraceae found extensively in Pakistan. (Batool, *et al.*, 2019). The only species abundantly found in the subtropical and tropical Himalaya regions is *Saussurea heteromalla* (Compositae), which has a large distribution that includes the Shivalik hills. In Hindi, it is known as Kaliziri, Murang and Batula. Three chemicals, arctiin, arctigenin, and chlorogenerin, have been isolated from *S. heteromalla* from Indian species. In earlier research, we looked for *in vitro* cytotoxic, antioxidant, antifungal, antibacterial and anti-diabetic properties in a crude methanolic extract of *S. heteromalla*. Among the activities carried out, a powerful cytotoxic impact on a human cervical cancer cell line (HeLa cells) was demonstrated, with the results being reported (Batool, 2022). *Saussurea* is a plant which is found in Asia, Europe and North America and contains more than 400 species. (Pandey *et al.*, 2007) In all over China about 300 species are approximately found as Tibetan medicines according to Chinese Pharmacopoeia (Yang *et al.*, 2010). The name of the plant is based on the Swiss philosopher Horace Benedict de Saussure (1740-1799) (Butola and Samant, 2010). *Saussurea lappa* is a well-known Indian medicinal plant that is used to treat inflammatory problems in traditional Indian medicine (Chandur, 2011). The present study was conducted to investigate phytochemical constituents present in methanolic flower extract of *Saussurea heteromalla* by using GC-MS. To investigate the anti-inflammatory, anti-oxidant, cytotoxic effect of *Saussurea heteromalla* methanolic flowers extract.

MATERIALS AND METHODS

Isolation and purification of Saussurea heteromalla flowers

Saussurea heteromalla was collected from Islamabad, Pakistan during the month of April and plant was identified from the herbarium of Quaid-i-Azam University, Islamabad, Pakistan with reference number (ISL-130608). The whole plant was shade dried and flowers of the *Saussurea heteromalla* were separated and grinded into the powder form. The percolator was used for extraction purpose. Dried powder about 2.5kg kept in a percolator for 15 days and 5liters of methanol was used as a solvent in which flowers were soaked. Methanolic extract was collected and concentrated through vacuum rotatory evaporator (Casuga *et al.*, 2016).

Chemicals and reagents

Carrageenan (Research-Organics), methanol (VMR-PROLABO), Dimethylsulphoxide (Duksan-Korea), 2,2-Diphenyl-1-picrylhydrazyl **(**DPPH) (Sigma-Aldrich), Ascorbic-Acid (Sigma-Aldrich), Diclofenac-Na (Sami-pharmaceuticals), Normal-saline (Revive-Pharmakon), Distilled water.

Anti-inflammatory activity

Carrageenan induced rat paw edema model was used. Six groups were made, each consisted of 5 rats. Group 1 rats were given 0.4% DMSO, which served as control. 1% carrageenan was given to Group II, which served as diseased group. Group III rats were given diclofenac sodium 10mg/kg dose intraperitoneally, one hour before carrageenan. Group IV, V, VI contain 100mg/kg, 200mg/kg, 400mg/kg p.o doses respectively for 5 days and on 5th day carrageenan was given intraperitoneally half an hour before the doses. (Morris, 2003) The inflammatory response in terms of volume changes was experimentally measured by plethysmometer (UGO BASILE S.R.L model-37215). (Gao *et al.*, 2019)

Anti-oxidant activity

DPPH method was used to evaluate anti-oxidant activity. DPPH is a free radical (Mensor *et al.*, 2001). 9.2ml DPPH was taken in 100ml of methanol. Ascorbic acid used as standard (positive control). Both are taken 1mg/ml dissolved in DMSO respectively.4mg of *Saussurea heteromalla* methanol and n-hexane flower extracts were calculated and then added 1ml DMSO in each extract respectively, stock solution of the both extract prepared.1µg/ml, 2µg/ml, 5µg/ml, 10µg/ml, 25µg/ml, 50µg/ml, 100µg/ml extracts were taken from stock solutions and filled the 96-well plate and in each well added the sufficient amount of DPPH solution up to 200µl. The well plate was covered through aluminum foil and kept for 60minutes. After 60minutes if the purple color changed to yellow, then antioxidant activity of extract carried out. Microplate reader was used to determine the absorbance of the extract at about 517nm (Hidayat *et al.*, 2017). For measurement of IC50 value the following formula was used (Batool *et al.*, 2019).

$$\text{Scavenaging effect} = \frac{\text{Absorbance of negative control} - \text{Absorbance of test sample}}{\text{Absorbance of negative control}} \times 100$$

Anti-cancer activity

Cell lines and cell culture conditions

Human malignant melanoma (HT144) cell lines were used which is skin cancer cell lines. Human malignant melanoma (HT144) cells were cultured in RPMI-1640 supplemented with 10% FBS,2mM L-glutamine,1mM Na-pyruvate, 100U/mL penicillin, 100µg/mL streptomycin at 37°C in a humidified 5% CO2 atmosphere (Arooj *et al.*, 2015).

Cell viability assay

The MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) test was used to assess the viability of the cells for the assessment of the cytotoxic effect of

the drug (Mosmann, 1983) The HT144 cells were inoculated into 96-well plates at a density of 15000 cells / well with 100µL of culture medium for the proliferation assay and were incubated for 24hours and then treated with different concentrations of the *Saussurea heteromalla* flowers extracts that is 0,6.25mg/ml, 12.5mg/ml, 25mg/ml, 50mg/ml, 100mg/ml, 200mg/ml, 400mg/ml. Incubated more for 24hours, in triplicates. The untreated cells were used for control purposes. Cells were washed 3 times with PBS after completion of treatment and supplied with fresh medium (100µL), followed by the addition of 20µL of MTT solution (5mg/mL) and incubated for 4hours. The culture media was removed and each well received 150µL DMSO and vibrated to completely dissolve the formazan crystals formed by living cells. Finally, using multiple microplate reader, the absorbance value corresponding to the living cells was calculated at 492nm (Arooj *et al.*, 2015).

$$\text{Cell viability (\%)} = \frac{\text{Absorbance value of experimental groups}}{\text{Absorbance value of control group}} \times 100$$

Ethical approval

The male Wistar albino rats (weight 150-200g each) were obtained from the animal house of Department of Pharmacology, Riphah Institute of Pharmaceutical Sciences Islamabad. The animals were kept under standard environmental conditions (25±2°C) in polypropylene cages under 12hours light and dark cycle each. Water and food were made available at libitium.

STATISTICAL ANALYSIS

The results were statistically analyzed by one way ANOVA, followed by post-hoc Tukey's test. The statistical analysis, creation of graphs and assessment was performed by using the statistical tool of Graph Pad Prism 6.

RESULTS

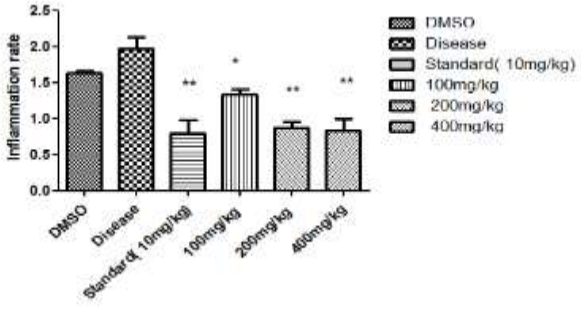

Fig. 1: The above graph shows the anti-inflammatory effect of methanolic extract at 100mg/kg, 200mg/kg, 400mg/kg doses, and diclofenac sodium at 10mg/kg dose. Data expressed as mean ± SEM (n=5). ***P* < 0.05, **P*<0.01 vs disease group, one -way ANOVA followed by post-hoc Tukey's test.

Anti-inflammatory activity

The inflammatory response in terms of volume changes was experimentally measured by plethysmometer. The standard that is diclofenac sodium and the methanolic extract of *Saussurea heteromalla* flower at 400mg/kg dose has equal anti-inflammatory action.

Anti-oxidant activity

Anti-cancer activity

MTT assay was used for cytotoxicity studies against HT144 cell lines.18.72% cell survive out of 100 when methanolic flower extract of *Saussurea heteromalla* was given at the dose of 400mg, which shows the cytotoxic effect.

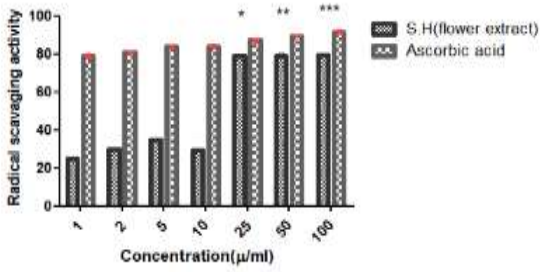

Fig. 2: The above graph shows the anti-oxidant effect of methanolic extract at 1µg/ml, 2µg/ml, 5µg/ml, 10µg/ml, 25µg/ml, 50µg/ml, 100µg/ml doses and Ascorbic acid (standard). Data expressed as mean ± SEM (n=3). ****P* <0.05, ***P*<0.01,**P*<0.001vs standard, One-way ANOVA with post-hoc Tukey's test.

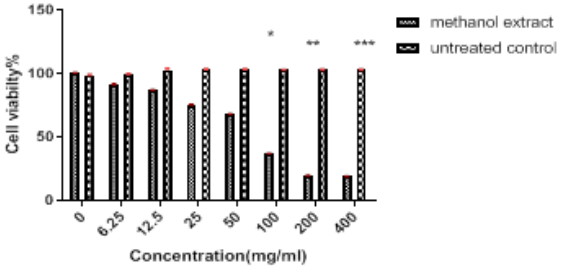

Fig. 3: The above graph shows the skin cancer activity of methanolic extract by using HT144 cell lines. Data expressed as mean±SEM (n=3). ****P*<0.05, ***P*<0.01,**P*<0.001vs untreated group, One-way ANOVA followed by post-hoc Tukey's test.

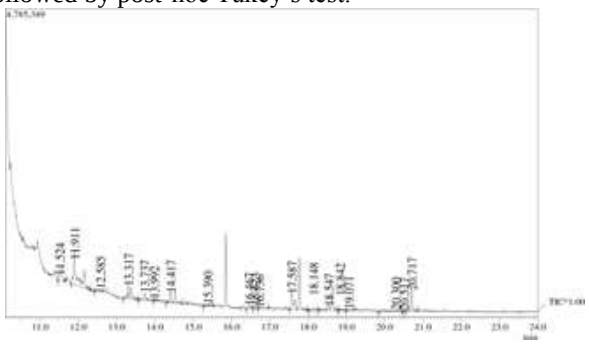

Fig.4: GCMS spectrum of *S. heteromalla* flower extract

Table 1: GCMS spectral peaks values

Peak#	R. Time	I. Time	F. Time	Area	Area%	Height	Height%	A/H
1	11.524	11.467	11.667	1011966	7.30	98953	5.12	10.22
2	11.911	11.817	12.292	3190941	23.01	385308	19.93	8.28
3	12.585	12.417	12.642	193401	1.39	31943	1.65	6.05
4	13.317	13.167	13.567	1068752	7.71	191978	9.93	5.56
5	13.737	13.567	13.967	527637	3.80	98648	5.10	5.34
6	13.992	13.967	14.042	41159	0.30	15934	0.82	2.58
7	14.417	14.292	14.717	1070561	7.72	149766	7.75	7.14
8	15.390	15.267	15.517	295880	2.13	30746	1.59	9.62
9	16.467	16.392	16.567	273520	1.97	66786	3.45	4.09
10	16.623	16.567	16.692	198349	1.43	48980	2.53	4.04

Table 2: Compounds names according to peaks in GCMS

No.	Name of compounds	Molecular Weight	Molecular Formula
1-	2-Amino-6,7-dimethyl-4-hydroxypteridine	191	$C_8H_9N_5O$
2-	Cyclohexylmethylbenzene	174	$C_{13}H_{18}$
3-	Hexanohydroxamic acid	131	$C_6H_{13}NO_2$
4-	N2,O-3',5'-Tris(trifluoroacetyl)deoxy guanosine	555	$C_{16}H_{10}F_9N_5O_7$
5-	Capraldehyde	156	$C_{10}H_{20}O$
6-	Heptanal	114	$C_7H_{14}O$
7-	9-Octadecynoic acid methyl ester	294	$C_{19}H_{34}O_2$
8-	cis-9-Octadecenamide	281	$C_{18}H_{35}NO$
9-	Dodecyl trifluoroacetate	282	$C_{14}H_{25}F_3O_2$
10-	Oxetane, 2-methyl-4-propyl-	114	$C_7H_{14}O$

DISSCUSION

Previous studies investigate the cytotoxic effect against HeLa cell lines and antibacterial and antifungal properties of *Saussurea heteromalla* was proven by a study. This study tested the anti-inflammatory, antioxidant and cytotoxic effect by using HT144 cell lines (Skin cancer cell lines) of *Saussurea heteromalla* flowers. Among the activities carried out, a powerful cytotoxic impact on a human cervical cancer cell line (HeLa cells) was demonstrated, with the results being reported Carrageenan induced rat paw edema model was used to evaluate anti-inflammatory activity of *Saussurea heteromalla* flowers and evaluated *in-vitro* antioxidant activity by DPPH method. anti-oxidant activity of methanolic extract is also very good. IC50 value of methanol was 25µg/ml. HT144 cell lines was used for cytotoxity studies through MTT assay. The inflammatory response in terms of volume changes was experimentally measured by plethysmometer. The standard that is diclofenac sodium and the methanolic extract of Saussurea heteromalla flower at 400mg/kg dose have equal anti-inflammatory action. In antioxidant activity DPPH method was used to evaluate anti-oxidant activity.in anti-cancer activity HT144 non-metastatic melanoma cell lines were used for cancer incidence assessment. Human malignant melanoma (HT144) cells were grown with 10 % FBS in RPMI1640, 2mM Lglutamine, 1mM Napyruvate, 100U/mL penicillin, 100µg/mL streptomycin at 37°C in a 5% CO2 humidified. (Syeda Arooj *et al.*, 2015). The MTT (3-[4, 5-dimethylthiazol-2-yl]-2, 5 diphenyl tetrazolium bromide) test was used to assess the viability of the cells and the cytotoxic effects of drugs often assessed by this method. In this assay living cells convert MTT into formazan which indicates the mitochondrial activity (Mosmann, 1983). Human fibroblast malignant melanoma (HT144) cell lines were used. The plating density was 15000cells/well. Treatment dose was (0-400mg) dissolved in DMSO. The exposure time was 24hrs. The calculations were relative to the untreated culture. 18.72% cell survive out of 100when methanolic flower extract of *Saussurea heteromalla* was given at the dose of 400mg/ml, which shows the cytotoxic effect, while n-hexane extract has very least cytotoxic effect that is 98% cell survive out of 100when n-hexane flower extract was given at the dose 400mg/ml. The bioactive components in the methanolic extract were determined and identified using a GC-MS method. 2-Amino-6,7-dimethyl-4-hydroxypteridine, Cyclohexylmethylbenzene, Hexanohydroxamic acid, N2,O-3',5'-Tris (trifluoroacetyl) deoxy guanosine, Capraldehyde, Heptanal, 9-Octadecynoic acid methyl ester, cis-9-Octadecenamide, Dodecyl trifluoroacetate and Oxetane, 2-methyl-4-propyl- are some compounds which are identified by GC-MS.

CONCLUSION

MTT assay, anti-inflammatory and *in vitro* anti-oxidant potential of methanolic extract of *Saussurea heteromalla* was used to evaluate its biological potential. GC-MS approach was done to determine and identify the bioactive compounds in the methanolic extract. 2-Amino-6,7-dimethyl-4-hydroxypteridine, cyclohexylmethylbenzene, hexanohydroxamic acid, N2,O-3',5'-Tris (trifluoroacetyl) deoxy guanosine, capraldehyde, heptanal, 9-octadecynoic acid methyl ester, cis-9-Octadecenamide, dodecyl trifluoroacetate and oxetane, 2-methyl-4-propyl- are some compounds which are identified by GC-MS. The biological profile, isolation and characterization-based investigations proclaiming the biologically leading active molecule which showed worthy activities that may lead to further investigation on other cancer cell lines.

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