Available online on www.ijppr.com
International Journal of Pharmacognosy and Phytochemical Research 2017; 9(8); 1159-1165

ISSN: 0975-4873

Research Article

The Study of Antioxidantion, Membrane Stabilization, Anti Protein-denaturation Property and Analysis of Phytochemicals in Three Species of *Tagetes* Leaf and Flower Extract

Puspal De[1*], Monidipa Dey[2], Madhumita J Mukhophadhyay[2]

[1]*Department of Genetics, Institute of Genetic Engineering. 30 Thakurhat Road. Kolkata- 700128,West Bengal, India.*
[2]*Department of Biotechnology, Institute of Genetic Engineering. 30 Thakurhat Road. Kolkata- 700128,West Bengal, India.*

Received: 14th April, 17; Revised 20th July, 17, Accepted: 15th Aug, 7; Available Online:25th Aug, 17

ABSTRACT

Since the ancient time nature provides several herbal phyto-chemicals for the beneficial of human. *Tagetes* sp is one of the common and valuable herbs with multiple uses. In the current study, qualitative screening of different phytoconstituents, anti protein denaturation and membrane stabilization property was checked in ethanolic extract of three different species of *Tagetes sp* namely *Tagetes petula, Tagetes tenuifolia, Tagetes erecta*. Three different concentration 50, 75, 100 microlitre were taken for membrane stabilization and anti protein denaturation method using 50 microgram/ml sodium diclofenec as a reference drug. The study revealed that flower and leaf extract from each sample have several bioactive compounds and significant membrane stabilization and anti protein denaturation property. Presence of several phyto-chemicals may help to scavenge the reactive oxygen, potent membrane destabilizing and anti protein denaturation agent in human. So, in future it could be possible to develop a new phyto derived drug for an inflammatory disease in human as literature review shows that the membrane destabilization and protein denaturation are the principal cause of inflammation related disease.

Keywords: *Tagetes* sp, membrane stabilization, anti protein denaturation, bioactive compound, flower and leaf extract.

INTRODUCTION

Use of selective herbs and plants as an alternative and complementary therapy is a category of medicine that includes a range of treatment approaches that fall outside the realm of conventional medicine. These traditional herbal medicines, also known as a good source of neutraceuticals, possess various beneficial effects on our health. In recent years an increasing amount of research regarding neutraceuticals is being done to establish the safety and efficacy of these therapies, though compared with mainstream medical therapies researches are still meagre.

As growing interest in these neutraceuticals or functional foods is leading to detailed research, it became absolute necessary to test the plants involved thoroughly for their different medicinal properties as neutraceuticals are natural compounds with bioactive properties having health promoting, disease preventing or curative properties[1]. It has been found that most of the observed therapeutic effects of plants are linked to their potent antioxidant activity. It is also said that, this kind of healing activity could be the traditional basis of plants used in Ayurveda.

Genus *Tagetes*, commonly known as marigold is used in Indian culture for different religious and decorative purpose. It was a native plant in Maxico and other warmer part of America and had been introduced in the Indian garden from long years back. It belongs to the family compositeae and generally orange, yellow and reddish in color[2]. The flowers contain several pigments but the color variations in different flowers of *Tageest spp* are mainly due to the presence of a specific pigment called lutein, a yellow plant pigment of carotenoid family. In many traditional native practitioners in northern India used these plans for the treatment of specific cancer and the leaves are specially used for their coagulating property. Literature review revealed that lutein, occurred in all green plants and also in many flower petals, has effectively inhibit oxidant induced cellular damage. Lutein has cytostatic activity on cancer cell line and accumulated in high level in human retina especially in macular region[3]. The flower used as natural food coloring agent and consumption seems to be safe and low risk of cardiovascular disease development, progression of cancer and formation of cataract[4-8]. The flowers also have medicinal importance against inflammation. Information from current review and traditional knowledge prompted us to check the qualitative and quantitative analysis of bioactive compounds, total antioxidant property, membrane stabilization property and anti protein denaturation property of the ethanolic extract of flower and leaves of three *Tagetes* species like *Tagetes petulla*,

**Author for Correspondence: puspal.dey@gmail.com*

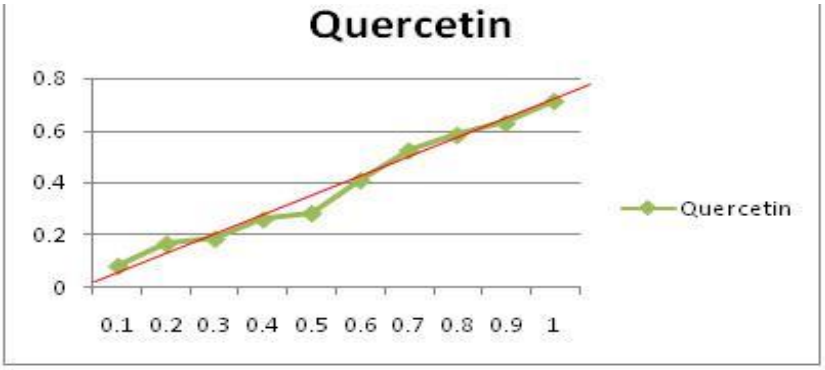

Figure 1: Reference Curve of Quercetin for Quantification of Flavonoids.

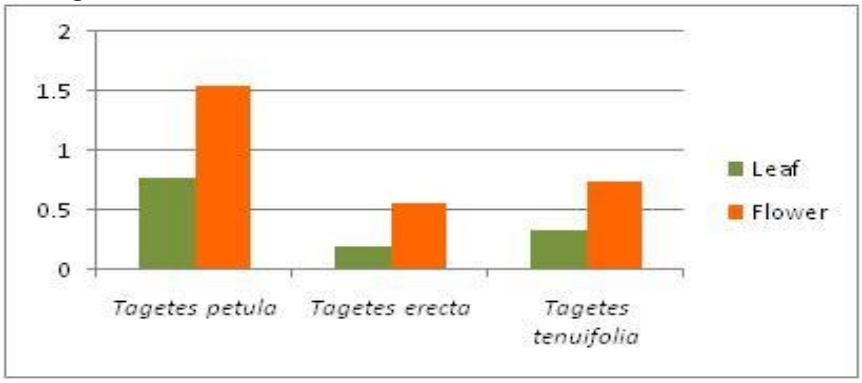

Figure 2: Quantification of Total Flavonoids in Three *Tagetes* spp from reference curve QE.

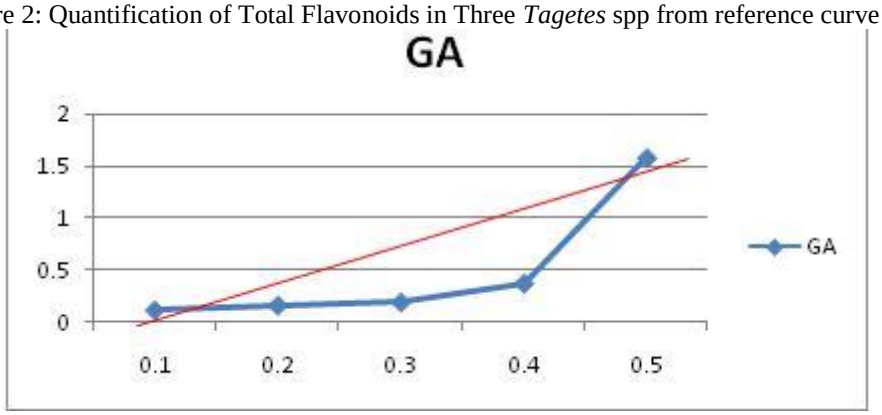

Figure 3: Reference Curve of Gallic acid for Quantification of Polyphenol.

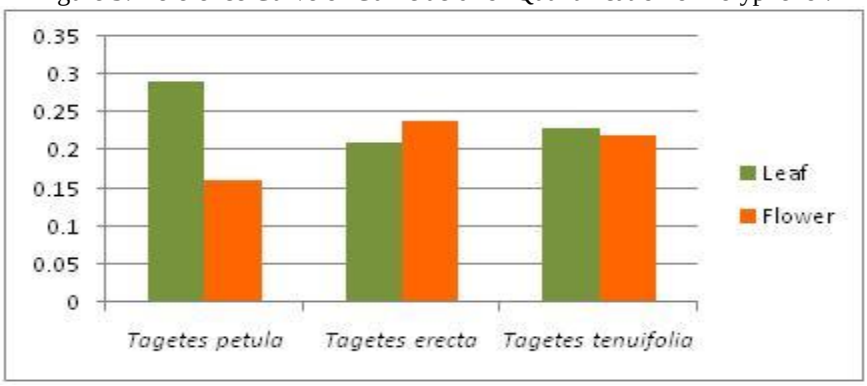

Figure 4: Quantification of Total Flavonoids in Three *Tagetes* spp from reference curve of GA

Tagetes erecta and *Tagetes teneuifolia*.

Herbal drugs are most common but herbalists usually extract plant substances in a different way the drug industry does. Herbalists believe that the remedy happens due to the delicate chemical balance of the whole plant, or mixtures of plants, not one particular active ingredient[9]. Believing in this principle we have taken the whole extract for the present study.

MATERIALS AND METHODS

Collection of Sample

The leaves and the flowers of several *Tagetes* species ware collected from the local market during the month of December and the plants were identified by Departmental Head. Dept. of Biotechnology. IGE.

Preparation of Extract

The samples were initially washed with tap water then with distilled water to remove soil, dust particles and other contaminants. They were dried at 37^0C in laboratory incubator. 50 gm of dried samples were dissolved in 200 ml of 100% ethanol and kept them 37ºC for another 72 hrs in a shaker incubator with 120 rpm. After that the samples were grinded and centrifuged with 2000 rpm for 5 minutes. The supernatant were collected to perform several test[10].

Qualitative analysis of phytochemicals

Bioactive compound or phytochemicals like tannin, flavonoids, steroid, phenols, amino acids, sterols and terpenoids ware determind by the different methods. The qualitative analysis was done by the standard method of Harbone[11].

Quantitative Estimation of Flavonoids

100µl flower and leaf extract from each sample were mixed with 100µl of 20% aluminium tricloride in methanol and a drop of acetic acid is added then the solution diluted with methanol up to 5ml. absorption at 415nm was read after 40 minutes. Acetic acid was used as a blank. The calibration curve was obtained by preparing Quercetin solutions in range of concentrations 0.1mg to 1.0mg/ml[12].

Quantitative Estimation of Polyphenol

100µl flower and leaf extract from each sample were dissolve in 900µl double distilled water. In this 1ml solution add 0.5 ml 2N of the Folin ciocalteu reagent and 1.5ml of 20% Na_2CO_3 solution was added, then the sample volume was made up to 8ml with distilled water fallowed by vigorous shaking and finally allowed to stand for 2 hrs and after that absorbance was taken at 765 nm. For standard calibration graph Gallic acid is used[13].

Quantitative Estimation of Total antioxidant

The total antioxidant activity of *Tagetes* was evaluated by phosphomolybdenum method where 0.3 ml of plant extract was mixed with a 3ml solution containing 0.6 mlo/L sulphuric acid, 28 mmol/L sodium phosphate and 47 mmol/L ammonium molybdate. The assay mixture with 100µl flower and leaf extract from each sample were incubated 95ºC for 90 minutes. After cooling at 25ºC absorbsence of the resulting solution was measured spectrophotometrically at 695 nm [14]. Ascorbic acid used as positive control. The percentage of total antioxidant was calculated by the following formula-

$\%= [(OD_{test}- OD_{blank})/(OD_{ascorbic\ acid}-OD_{blank})]X100$

In vitro Membrane stabilization assay

To study the anti-inflammatory activity, the HRBC membrane stabilization method was adopted after Gandhisan 1991 [15]. Fresh blood was collected from healthy donors without the history of NSAIDS administration for at least two weeks prior to the experiment. The equal volume of sterilized Alsever solution and blood were mixed, the mixture was centrifuged at 3000 rpm and packed cell were washed twice with isosaline. The washed packed cell was made a 10% (v/v) suspension with isosaline to make a HRBC suspension. The assay mixture contained 1ml PBS (pH 7.4), 2ml of hyposaline (0.36% KCL), 0.5ml HRBC suspension and 1 ml of Test solution. Sodium diclofenec was used as reference drug and distilled water was used as control. All the assay mixtures were incubated at 37ºC in an incubator for 30 minutes and after that centrifuged at 1000 rpm for 2 minutes. The supernatant containing haemoglobin was estimated using spectrophotometer at 560 nm. 50mg sodium diclofenec was didssolve in 50ml double distilled water and the final solution was used as reference drug solution.

The percentage inhibition of membrane stabilization was calculated by using the following formula

$\%\ \text{inhibition} = 100 \times [OD_1\text{-}OD_2/OD_1]$

Where, OD_1 = absorbance of Control, OD_2 = absorbance of test sample

In vitro Protein Denaturation Assay

In this experiment 0.2 ml of egg albumin (from fresh hen's egg) act as a protein source, 2.8 ml of phosphate buffered saline (PBS, pH 6.4) and 2 ml of varying concentrations of the test extract such as 50µl, 75µl, 100µl ethanolic extract of flower and leaf extract from each sample were mixed to prepare assay mixture. Similar volume of double-distilled water served as control. Then the mixtures were incubated at 37±2ºC in a BOD incubator for 15 minutes and then heated at 70ºC for 5 minutes in water bath. After cooling, their absorbance was measured at 660 nm by using vehicle as blank. Diclofenac sodium at the final concentration of (50µg/ml,) was used as reference drug and treated similarly for determination of absorbance[15-16].

The percentage inhibition of protein denaturation was calculated by using the following formula

$\%\ \text{inhibition} = 100 \times [\ Vt / Vc - 1]$

Where, Vt = absorbance of test sample, Vc = absorbance of control

RESULT

Result of Qualitative analysis of phytochemical

In the present study, qualitative analysis of ethanolic extract of leaf and flower of several *Tagetes* species was carried out. In the experimental results shows that tannin, flavonoids, steroids, phenols and amino acids were present in the extracts but terpenoids and sterols were absent and the results are depicted in the Table-1 elaborately.

Result of Quantitative Estimation of Flavonoids

Ethanolic extract of three *Tagetes* species shows presence of flavonoids and the concentration of flavonids were more in flowers than the leaves extract in all three species. The *Tagetes petula* flower shows maximum amount of flavonoid content (1.54±0.17 mg/ml equivalent to quecertin) and the *Tagetes tenuifolia* flower shows moderate amount of flavonoid content (0.74±0.47 mg/ml equivalent to quecertin) and *Tagetes erecta* flower shows minimum amount of flavonoid content (0.56±0.16 mg/ml equivalent to quecertin). Similarly the leaf extract shows

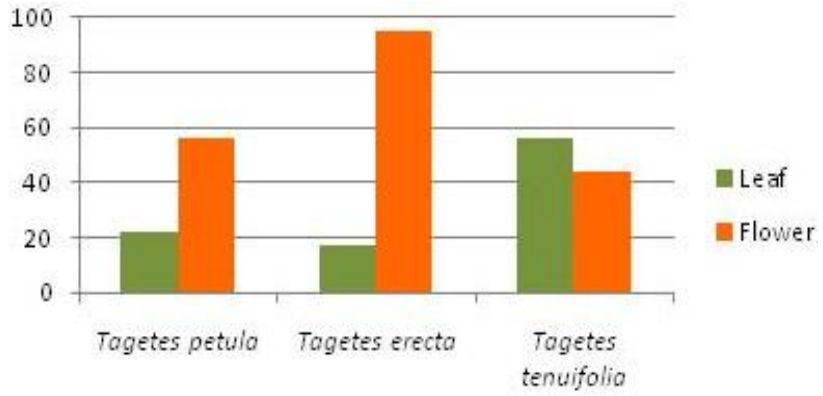

Figure 5: Quantification of Total antioxidant property in Three *Tagetes* spp.

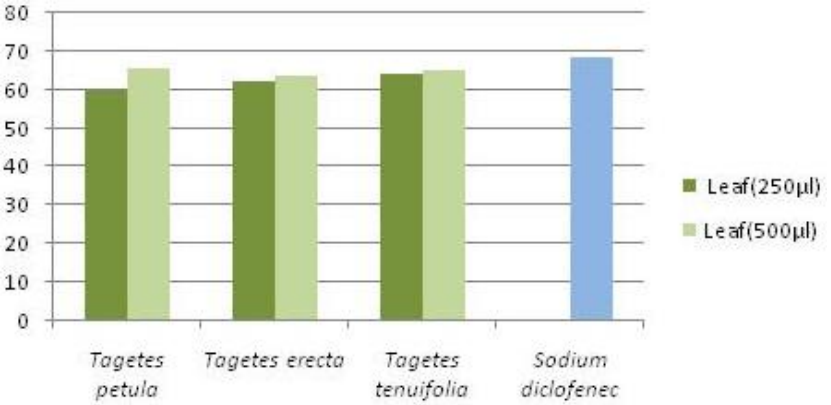

Figure 6: *In vitro* membrane stabilization activity of the ethanolic leaf extract of Three *Tagetes* spp using Sodium diclofenec as reference drug.

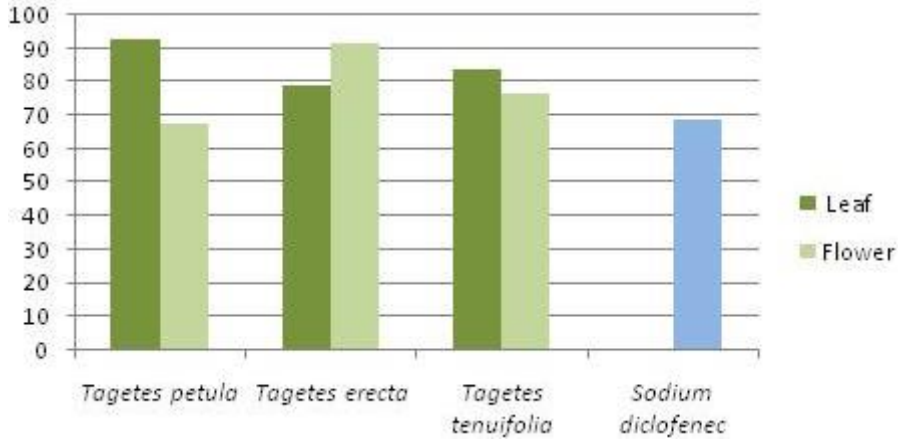

Figure 7: *In vitro* Protein Denaturation activity of the ethanolic leaf extract of Three *Tagetes* spp.

maximum (0.78±0.11 mg/ml equivalent to quecertin), moderate (0.34± 0.08 mg/ml equivalent to quecertin) and minimum (0.20±0.23 mg/ml equivalent to quecertin) flavonoid content respectively. Reference Curve of Quercetin for Quantification of Flavonoids and Quantification of Total Flavonoids in Three *Tagetes* spp from reference curve of QE were depicted in Figure-1 and Figure-2.

Result of Quantitative Estimation of Polyphenol

All The three *Tagetes* species shows presence of polyphenols in both the flowers and the leaves extract. The *Tagetes petula* leaf extract shows maximum amount of polyphenol content (0.29±0.57 µg/ml equivalent to Gallic Acid) and the flower extract shows minimum amount of polyphenols (0.16±0.36 µg/ml equivalent to Gallic Acid) among the all three species. In the *Tagetes tenuifolia* both the flower and leaf extract shows moderate amount of polyphenol content (0.23±0.44 µg/ml equivalent to Gallic Acid) and (0.22±0.63 µg/ml equivalent to Gallic Acid) respectively. *Tagetes erecta* flower shows maximum amount of polyphenol content (0.24±0.26 µg/ml equivalent to Gallic Acid). Similarly the leaf extract shows minimum amount of polyphenol content (0.21±0.71 µg/ml equivalent to Gallic Acid). Reference Curve of Gallic acid for Quantification of Polyphenol and Quantification of Total polyphenols in Three *Tagetes* spp from reference curve of GA were depicted into Figure-3 and Figure-4.

Result of Quantitative Estimation of Total antioxidant

Table 1: Qualitative analysis of phytochemicals of the ethanolic leaf and flower extract of Three *Tagetes* spp.

Determination Test	Procedure	Result
Tanin Test	Lead acetate ssolution was added with sample extract and curry white precipited observed	Positive
Flavonoid Test	Concentrated sulphuric acid was added with sample extract and the color was changed to orange color	Positive
Steroid Test	Chloroform and conc. Sulphuric acid were added with sample extract slowly with the side of the test tube and observed brown upper layer.	Positive
Phenol Test	5% ferric chloride solution was added with sample extract and dark green color was observed.	Positive
Amino acid Test	Add 10% ninhydrine solution and incubate at 60^{o}C in water bath. The dark violet color observed	Positive
Sterol Test	Concentrated sulphuric acid was added with sample extract and no change of color was observed	Negetive
Terpenoid Test	Add distilled water and shake vigorously. No brown color interface observed	Negetive

Table 2: *In vitro* membrane stabilization activity of the ethanolic leaf extract of Three *Tagetes* spp.

Treatment	Concentration(µl)	% Inhibition
Control	----------	----------
Tagetes petula	250	59.8
leaf	500	65.6
Tagetes erecta	250	62.1
leaf	500	63.8
Tagetes	250	64.0
tenuifolia leaf	500	65.3
Sodium Diclofenec	50	68.8

Percentage of total antioxidant property was found highest among flower extract in *Tagetes erecta* flower (95%) then *Tagetes petulla* flower (55%) followed by *Tagetes tenuifolia* flower (44%). Among leaves extact, *Tagetes tenuifolia* has been shown highest antioxidant property (56%) then *Tagetes petulla* leaf (22) followed by *Tagetes erecta* leaf (16.7%). The result was graphically represented in Figure-5.

Result of In vitro Membrane stabilization assay

All the three species of *Tagetes* leaf extract has been shown a good percentage of inhibition against hypotonicity induced membrane destabilization but the concentration of extract used for the experiment lead the result. In this experiment two variable concentrations (250µl and 500µl) of same leaf extract were used. As shown in the result, *Tagetes patella* 500 µl leaf extract shows highest percentage of inhibition but 250ul of same extract shows lowest percentage of inhibition and the *Tagetes erecta* and *Tagetes tenuifolia* leaves shows moderate inhibition percentage . Surprisingly flower extract of all the three *Tagetes* species did not have any percentage of inhibition against hypotonicity induced membrane destabilization. The result and concentration of different extracts and drug were depicted in Table-2 and the graphical representation of the result was shown in Figure-6.

Result of In vitro Protein Denaturation Assay

All the three species of *Tagetes* leaf extract as well as flower extract has been shown a good percentage of inhibition against heat induced protein denaturation. *Tagetes patella* leaf extract shows highest percentage of inhibition but flower extract shows lowest percentage of inhibition and the *Tagetes erecta* and *Tagetes tenuifolia* leaves and flower extract shows moderate inhibition percentage against heat induced protein denaturation. The main noticeable thing in this case is that the Tagetes leaves extract have strong protein denaturation property and it act as a very minute concentration, because after huge dilution of crude extract like 1:15= extract: DH_2O have the potent anti protein denaturation activity. The result and concentration of different extracts and drug were depicted in Table-3 and the graphical representation of the result was shown in Figure-7.

DISCUSSION

Inflammation is common feature of several diseases. It mainly occurs due to the endotoxin released by the pathogen during pathogenic infection, due to sudden physical shock or accident and also associated with many other diseases. Inflammation mainly is of two types- acute and chronic, but both types are painful and create many difficulties in daily lifestyle. Inflammations are not always fatal like cancer and cardiovascular diseases but it occurs in almost same frequency with these two fatal diseases[17-18]. According to the severity it was not properly ranked but medical practitioner and clinician throughout the world faces difficulty to treat the patients with inflammation and inflammation related disease.

The most common and popular remedy from inflammation and inflammation related disease is administration of oral or intramuscular non steroidal anti inflammatory drug. But it has huge side effect as prolong use of NSAID may cause other disease like ulcer, cancer, renal failure, allergy, skin irruption, digestive problems and so on[19-20]. Alternative medicine or herbal medicine is largely needed at that point. In the modern age, many drugs are prepared from medicinal plants and plant parts and the clinician sometimes switch to herbal therapy and neutraceutical therapy rather than pharmaceutical approach for better performance of pain relief.

ROS or reactive oxygen species which are the naturally produced reactive molecules within the body as 5% of inhaled oxygen converted to these ROS during metabolism. Physical or mental stress factor and over workload, excess physical exercise, hormonal imbalances

Table 3: *In vitro* Protein Denaturation activity of the ethanolic leaf and flower extract of Three *Tagetes* spp.

Treatment	Concentration	% Inhibition
Control	----------	----------
Tagetes petula leaf	1:15(Extract: DDH_2O)	92.6
Tagetes petula flower	1:15(Extract: DDH_2O)	67.6
Tagetes erecta leaf	1:15(Extract: DDH_2O)	79.0
Tagetes erecta flower	1:15(Extract: DDH_2O)	91.6
Tagetes tenuifolia leaf	1:15(Extract: DDH_2O)	83.8
Tagetes tenuifolia flower	1:15(Extract: DDH_2O)	76.4
Sodium Diclofenec	50µg/ml	68.74

some time produces the ROS molecules. Electronic configuration in its outer orbits is the reason for the strong oxidizing power. Due to strong oxidizing nature it can accelerate cell mortality and cell damage by easy reaction with cellular constituents. Many antioxidant molecules are produced within the body which reduced the cellular damage caused by ROS with scavenging method. In normal homeostasis mechanism ROS molecules are completely scavenge by the antioxidant produced by the body. When the balance between ROS and antioxidant was disturbed then the problem arises and the residual ROS molecules then leads to many stress related disease[21-24].

ROS react with membrane lipids causing cellular membrane destabilization which in-turn releases cellular materials within the intracellular space and finally inflammation occurred. These molecules also react with membrane protein or other cellular or sub cellular proteins and performed conformational change in protein structure which finally lead to protein denaturation. Denaturation and renaturation is a common characteristic of protein and during denaturation the functional properties may get lost, but in case of removal of denaturing factors it renatures again and gets full functional conformation back. Sometimes removal of the denaturing factors does not give back its full conformation which is called permanent protein denaturation. In these situations finally the denatured proteins get precipitated and removed from the specific site by the macrophages. Excess deposition of denatured protein and aggregation of macrophages at this particular site is another cause of inflammation[25.] So, if the cellular membrane remains stable then no release of cellular materials within the intracellular space occurs and no occurrence of inflammation happens. Thus increase of membrane stabilization property is the main way out of this problem. Finally, the lower the denaturation of cellular proteins, the rate of inflammation also gets reduced.

Plants have several bioactive compounds or phytochemicals like poly-phenols, flavonoids, tannin, steroids, which have potent antioxidant property. They may work individually or collectively to scavenge the reactive oxygen species and if these compounds are able to reduce ROS molecules then they must be also have strong membrane stability and potent anti-protein denaturation activity which will be able to reduce the inflammation. So, plant extract with potent antioxidant property, membrane stabilization property, anti protein denaturation property have great medicinal value to produce the alternative medicine which may be used as potential anti-inflammatory drug in recent future. In pharmaceutical research the pharmacist mainly devoted to isolate pure products for the production of new drug but the herbalist mainly believe in combinatorial product for drug designing because balance of all chemical compounds in a particular combination may act properly and more effectively as they can act as individually thus they use plant extract instead of specific compound[26-27].

In the current study, three different species of *Tagetes* leaf and flower extract were used as concentration dependant manner, to evaluate the preliminary anti inflammatory property of the plant by membrane stabilization method, and protein denaturation assay. To explain or understand the actual mechanism, estimation of total antioxidant, poly-phenols, flavonoids and determination test of other bioactive compounds were done with the same extract.

CONCLUSION

In the present study, preliminary in vitro anti-inflammatory property was evaluated by membrane stabilization methods and protein anti-denaturation method in three different *Tagetes* species namely *Tagetes petula, Tagetes erecta, Tagetes tenuifolia*. To know the mechanism behind these anti-inflammatory properties several other biochemical tests were done such as qualitative analysis of bioactive compounds, quantitative analysis of flavonoids and polyphenols, estimation of total antioxidant property. Both the flower and leaf ethanolic extract were used for the study. Comparative analysis within the three species of *Tagetes* shows that the species *petula* have the slightly better result within the experiments performed than the species *erecta* and *tenuifolia*. The anti-inflammatory and antioxidant effect of this plant should be further evaluated to confirm the bioactive compounds responsible for these activities in pursuit of newer phytotherapeutics against inflammatory diseases and many oxidative stress related disease.

ACKNOWLEDGEMENT

The authors thankfully acknowledge the Director and Vice Principal of Institute of Genetic Engineering for funding and affiliation. We are also thankful to all laboratory members and other associated person of IGE for their enthusiastic participation.

REFERENCES

1. H. Nasri, A. Baradaran , H. Shiraz, MR. Kopaei. New concept in neutraceuticals as alternative for pharmaceuticals. Int J Prev Med, 5(12)(2014), pp 1487-1499.
2. NT. Nehr. The ethnobotany of tagetes. Econ Bot., 22(1968), pp 317–324. doi: 10.1007/BF02908126. [Cross Ref].
3. W. Gau, HJ. Plosche, C. Wunsche. Mass spectrophotometric identification of xanthophylls fatty acid esters from marigold flowers (*Tagetes erecta*) obtained by high performance liquid performance liquid chromatography. J Chromatogr, 262 (1983), pp 277–284. doi: 10.1016/S0021-9673(01)88106-1. [Cross Ref].
4. DE. Breithaupt, J. Schlatter. Lutein and Zeaxanthin in new dietary supplements-analysis and quantification. Euro Food Res Technol, 220 (2005), pp 648–652.doi: 10.1007/s00217-004-1075-2. [Cross Ref].
5. BP. Chew, MW. Wong, TS. Wong. Effects of lutein from marigold extract on immunity and growth of mammary tumours in mice. Anticancer Res, 16(16B) (1996), pp 3689–3694.[PubMed].
6. LX. Zhang, RV. Coney, JS. Betram. Carotenoids enhance gap functional communication and inhibit lipid peroxidation in C3H/10T/2 cells: relationship to their cancer chemo preventive action. Carcinogenesis, 12(1991), pp 2109–2114. doi: 10.1093/carcin/12.11.2109. [PubMed] [Cross Ref].
7. KR Martin, ML. Eailla, JC. Smith. β-Carotene and lutein protect HepG2 human liver cells against oxidant-induced damage. J Nutr, 126(1996), pp 2098–2106. [PubMed].
8. LA Fullmer, A. Shao. The role of lutein in eye health and nutrition. Am Asso Cereal Chem, 46(2001), pp 408–413.
9. P. Luhn, J. Walker, M. Schiffman, RE. Zuna, ST. Dunn *et. Al* The role of co-factors in the progression from human papilloma virus infection to cervical cancer. Gynecologic Oncology, 128 (2)(2013), pp 265–270.
10. P. De, S. Sarkar, MJ. Mukhopadhyay. Screening the bioactive compound and analyze the membrane stabilization property of *Vitex negundo* aqueous and alcoholic extract, In J Pharmacgnosy Phar Res, 9(1)(2017), pp 76-78.
11. AJ. Harbone. Phytochemical methods: a guide to modern technique of plant analysis Chapman Hall, New York (1973).
12. Z. Jia, M. Tang, J Wu. The determination of flavonoid contents in mulberry and their scavenging effects on superoxide radicals. Food Chem 1999; 64(1999), pp 555-559.
13. VL. Singleton, Jr JA.Rossi. Colorimetric of total phenolics with phosphomolybdic-phosphotungstic acid reagents Am J Enol Vitic, 16(1965), pp 144-158.
14. P. Prieto, M. Pineda, M. Aguilar. Spectrophotometric quantitation of antioxidant capacity through the formation of a phosphomolybdenum complex: specific application to the determination of vitamin E. Anal Biochem, 269(2)(1999), pp 337-341.
15. R. Gandhudasan, A. Thamaraichhelvan, S. Babraj. Anti-inflamma-tory action of Lannea cormandelica by HBRC membrane stabilization. Fitoterapia, LXII(1991), pp 81-83.
16. E. Umapathy, EJ. Ndebia, A. Meeme, B. Adam, P. Menziwa, BN. Nkeh-Chungag, JE. Iputo. An experimental evolution of Albuca setosa aqueous extract on membrane stabilization, protein denaturation and white bloob cell migration during acute inflammation. J Med Plant Res, 4(9)(2010), pp 789-795.
17. H. John. Guyton and Hall textbook of medical physiology (12th ed. ed.). Philadelphia, Pa.: Saunders/Elsevier(2011) p. 428. ISBN 978-1-4160-4574-8.
18. F. Colotta, P. Allavena, A. Sica, C. Garlanda, A. Mantovani, S. Allavena, G. Mantovani. Cancer-related inflammation, the seventh hallmark of cancer: links to genetic instability. Carcinogenesis (review), 30 (7)(2009), pp 1073–81.doi:10.1093/carcin/bgp127. PMID 19468060.
19. J Lin, W. Zhang, A. Jones, M. Doherty. Efficacy of topical non-steroidal anti-inflammatory drugs in the treatment of osteoarthritis: meta-analysis of randomised controlled trials. BMJ,(2004), pp 329:324.
20. LS. Simon, LM. Grierson, Z. Naseer Z, *et al*. Efficacy and safety of topical diclofenac containing dimethyl sulfoxide (DMSO) compared with those of topical placebo, DMSO vehicle and oral diclofenac for knee osteoarthritis. Pain, (2009), pp 143:238.
21. B. Gillham, K. Papachristodoulou, JH. Thomas. Wills' Biochemical Basis of Medicine. Oxford: Butterworth-Heinemenn, (1997), pp. 351.
22. U. Bandyopadhyay, D. Das, PK. Banerjee. Reactive oxygen species: Oxidative damage and pathogenesis. Curr Sci. 77(1999), pp 658.
23. DA. Lewis. Anti-inflammatory Drugs from Plants and Marine sources. Basel: Birkhauser Verlag, (1989), pp35.
24. RS. Cotran, V. Kumar, SL Robbins, Pathologic Basis of Disease. PhiladelphiaW.B. Saunders Company, (1994), pp. 73.
25. JY. Chang. Conformational isomers of denatured and unfolded proteins: methods of production and applications. Protein J, 28(1)(2009), pp 44-56.
26. J Sadique, NA. Al-Rqobah, MF. Bughaith, AR. El-Gindy. The bioactivity of certain medicinal plants on the stabilization of RBC membrane system. Fitoterapia LX(1989), pp 525-532.
27. OP. Tiwari, YB. Tripathi. Antioxidant properties of different fractions of Vitex negundo Linn, Food Chem, 100(2007), pp 1170–1176.