Original Article

Effect of *Ocimum sanctum* in Sodium fluoride (NaF) induced Fluorosis in Rats: A Study with Respect to Antioxidant Enzymes and Fluorosis Markers

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ABSTRACT

Introduction: Fluoride contamination was observed in groundwater's of India, Pakistan, China, Indonesia and several other countries. In India the states like Andhra Pradesh, Telangana, Rajasthan, Haryana etc are contaminated with fluoride. Approximately 68 million people are suffering from fluorosis in India. The current study was done to know the antioxidant effect, anti-fluorosis and free radical scavenging property of *Ocimum sanctum* (OS). **Methods:** Superoxide dismutase (SOD), glutathione peroxidase (GPx), glutathione (GSH), glutathione reductase (GR), glutathione-s-transferase (GST), catalase (CAT), malondialdehyde (MDA), alanine aminotransferase (AAT), alkaline phosphatase (ALKP), aspartate aminotransferase (AST) and flurosis markers phosphorus (P_4) and calcium (Ca) are estimated in all groups. 1,1-diphenyl-2-picryl hydrazyl radical (DPPH), hydroxyl radical, hydrogen peroxide (H_2O_2), Gas chromatography–mass spectrometry (GC-MS) was analyzed in methanolic extract of *Ocimum sanctum*. **Results:** SOD, GR, GSH, GPx CAT, activities and Ca levels are decreased in sodium fluoride (NaF) intoxicated rats. Whereas MDA, ALKP AST, AAT, and P_4 levels are increased in NaF rats. However, *Ocimum* phenolic fraction treatment normalized the antioxidant enzymes, hepatic markers and flurosis markers in NaF intoxicated rats. DPPH, H_2O_2 and hydroxyl radical of ocimum posses potent free radical scavenging activities. GC – MS studies of *Ocimum sanctum* showed the presence of many bioactive compounds like Caryophyllene oxide, 2-(2-Propenyl)-m-anisidine, 2H-Pyan-2-one, 5,6-Dihydro-4-(2-methyl-3-methylene-1-buten-4-yl etc. Further, histopathological observations prove that ocimum protects the hepatic tissue from NaF intoxication. **Conclusion:** Our study proves that *Ocimum sanctum* phenolic fraction protects the hepatic tissue from Na F induced fluorosis in rats.
Key words: *Ocimum sanctum*, Antioxidant Enzymes, Fluorosis markers, Liver markers, GC-MS analysis, Rats.

INTRODUCTION

Fluorosis is a worldwide health problem resulting from excess consumption of fluoride through food products, ingesting water, and industrial contaminants over a long period. Fluorosis affects teeth, liver, bone, kidney and other parts of human body.[1,2] The major complications of fluorosis are dental fluorosisand skeletal fluorosis.

The practice of herbs to cure diseases was observed, since the dawn of civilization. Plant derived drugs are used to treat diseases like cancer, hepatitis, diabetes etc. The World Health Organization reported that traditional medicines like Ayurveda, sidda, unani use plant drugs to treat many health problems, because they are cheap and have no side effects.[3,4]

Ocimum sanctum is known as Tulasi. *Ocimum sanctum* has antiseptic, antimicrobial, anti-bactericidal, anti-inflammatory, antioxidant, antiulcer, anti-diarrheal and anti-diabetic

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properties.[5-8] However, no study was carried on the anti-fluorosis property of *Ocimum sanctum*.

The aim of current study was to explore the anti-fluorosis, antioxidant activity of phenolic fraction of *Ocimum sanctum* in NaF toxicity rats. Our study is the first reported research investigation on the effect of phenolic fraction of *Ocimum sanctum* in fluorosis intoxicated rats.

MATERIALS AND METHODS

Collection of plant material and Preparation of extracts

Fresh and good *Ocimum sanctum* leaves are collected from Tirupati, A. P, India. The methanol extract of OS leaves are prepared as per the experimental protocol and was used for the study of free radical scavenging assay and GC MS analysis.

Chemicals and reagents

The chemicals are purchased from Himedia, Merck and Sigma–Aldrich.

Extraction of Phenols from *Ocimum Sanctum*

Phenolic fraction was extracted from *Ocimum sanctum* by the method of Magalhães *et al.* 2010.[9]

Treatment Groups

The young rats are divided into five groups.

Normal Control - Group I (NC): Six rats were treated with saline (0.9% NaCl) for the period of 60 days.

Ocimum Control - Group II (Ot): Six rats were treated with *Ocimum sanctum* phenolic fraction (100mg/kgBW) for the period of 60 days.

NaF treatment - Group III (Na F): Six rats were treated with sodium fluoride (10mg/kgBW) dissolved in water for the period of 60 days.

NaF + Ocimum treatment - Group IV (NaF+Ot): Six rats were treated with fluoride and Ocimum phenolic fraction for the period of 60 days.

Na F+ Vitamin treatment - Group V (NaF+Vit C): Six rats were treated with fluoride and Vitamin C for the period of 60 days.

After completion of last treatment i,e after sixty days treatment the rats are sacrificed by cervical dislocation and the liver tissue was collected in ice cold condition at 4°C. The liver tissues are stored in deep freezer at -80°C and later liver tissue was used for the estimation of antioxidant enzymes and other parameters. Blood sample was collected and used for analysis of liver markers and fluorosis makers.

Estimations of Antioxidant Enzymes

The antioxidant enzymes like superoxide dismutase (SOD), glutathione reductase (GR), glutathione –s–transferase (GST), glutathione (GSH), catalase (CAT), glutathione peroxidase (GPx), activities, and MDA levels were estimated by the methods of Misra and Fridovich,[10] Carlberg and Mannervik,[11] Akerboom and Sies,[12] Habig *et al.*,[13] Aebi,[14] Flohe and Gunzler,[15] Ohkawa *et al.*,[16] respectively.

Liver markers AST, ALT and ALP activities and Fluorosis markers Calcium, phosphorus levels are measured by standard kits.

Estimation of Free radical scavenging activity

DPPH, hydroxyl radical and hydrogen peroxide (H_2O_2) radical activities of ocimum are estimated by the methods of Koleva *et al.*,[17] Halliwell *et al.*[18] and Rosen and Rauckman.[19]

GC-MS Analysis for Bioactive compounds in *Ocimum sanctum*

The phytochemical compounds like polar, semi polar, nonpolar and other compounds are present in *Ocimum sanctum* are recognized by GC- MS instrument. Gas Chromatography (GC) peaks obtained for compounds in this study are coupled with Mass Spectroscopy (MS) and compound structures present in ocimum are searched in National Institute of Standard and Technology (NIST) library and their structures are drawn by using Marvin Sketch a Chemo informatics (*in silico*) tool.

Histopathological studies

Liver tissue was immediately fixed in 4 % paraformaldehyde phosphate buffer solution, after collection and process, it is used to assess the histopathology.

Statistical study

The results of our study are expressed as mean values ± standard deviation. Statistical comparison was carried with five groups by using one-way ANOVA with Dunnett's multiple comparison Test. Differences are considered significant at $p < 0.01$.

RESULTS

Free radical scavenging assay of *Ocimum sanctum*

DPPH activity expressed by methanolic extract and ascorbic acid are depicted in Figure 1. In the current study, *Ocimum* at the estimated concentrations showed significant DPPH scavenging activity, which shows free radical scavenging activity of *Ocimum sanctum*.

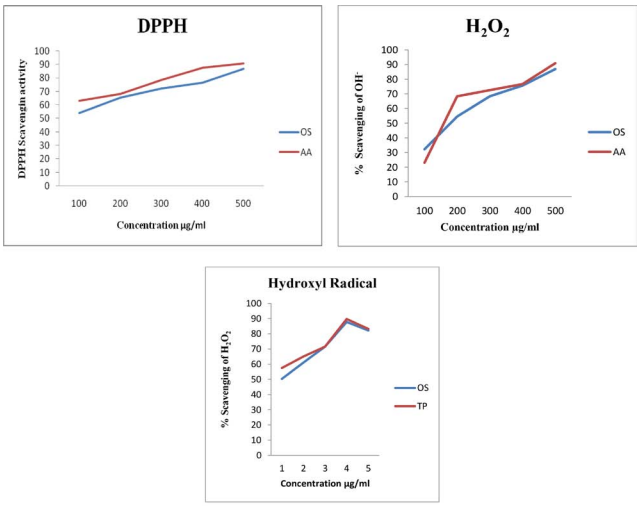

Figure 1: Free radical scavenging activity (DPPH, Hydrogen peroxide and Hydroxy Radicals) of metaholic extract of *Ocimum sanctum* with ascorbic acid and α tocopherol as standards.

The deoxyribose assay is a used to determine the hydroxyl radical activity of OS at different concentrations. (Figure 1). In the current study *Ocimum sanctum* at different concentrations posses significant hydroxyl radical scavenging potential.

Our results represents that the H_2O_2 scavenging activity of OS decreases than that of α-tocopherol. Hydrogen peroxide converts to water due to the antioxidant compounds in the methanolic extract of OS.

GC–MS Analysis for phytochemical profile identification

GC–MS can discover many chemical compounds in the plants, food, and other samples. GC–MS analyse the structure of different bioactive compounds. In our analysis, we reported 14 compounds in *Ocimum sanctum* methanolic extract. GC–MS chromatogram reported the presence of bioactive compounds like 2H-Pyan-2-one, 5,6-Dihydro-4-(2-methyl-3-methylene-1-buten-4-yl, Tricyclo[6.3.3.0] tetradec-4-ene, 10,13-dioxo, Perhydrohistrionicotoxin, 2-methylthio-2-depentyl,Trans-Z-à-Bisaboleneepoxide,2-(2-Propenyl)-m-anisidine, Patchoulene, 1,2,4-Triazol-4-amine 5-methyl-3-(3,5-dimethylpyrazol-1-yl)-Naphthalene,1,2,3,4,4a,5,6,8a-octahydro-4a,8-dimethyl-2-(1-methylethenyl-, [2R-(2à,4aà,8aà)], Caryophyllene oxide, 9-Octadecenoic acid [Z], (tetrahydro-3-furanyi)methyl ester, 5-methyl-3-(3,5-dimethylpyrazol-1-yl), 7,10-Octadecadienoic acid, methyl ester, Z-10-Methyl-11-tetradecen-1-ol propionate, Nonadecanoic acid, 18-oxo-, methyl ester, 4-Piperi-dineacetic acid, 5-ethylidene-2-[3-(2-hydroxyethyl)-1H-indol-2-yi]-à methylene-, methyl ester, [2S-(2à, 4à, 5E)]. (Figure 2, 3 and Table 1).

Effect of Phenolic fraction of *Ocimum* on Hepatic antioxidant enzymes in Sodium Fluoride treatment rats

In the current investigation, SOD, GPx, GR, CAT, activities, GSH levels are decreased in NaF intoxicated rats. However, with phenolic fraction of Ocimum supplementation in Na F rats, we observed significant elevation. But MDA content and GST activity are significantly increased in NaF intoxicated rats, however Ocimum supplementation decreased GST activity and MDA content in NaF rats. This shows the potential antioxidant effect of *Ocimum* (Figure 4).

Effect of Phenolic fraction of *Ocimum* on Serum and Fluorosis markers in Fluorosis rats

In the present study, AST, ALT, ALP activities and phosphorus levels are elevated in sodium fluoride intoxicated rats. But Calcium levels depleted in NAF rats. This indicates severe liver injury in Fluorosis condition. However phenolic fraction of Ocimum for 60 days supplementation significantly altered all the liver parameters and fluorosis markers in fluorosis rats. (Table 2).

Effect of Phenolic fraction of Ocimum on Liver tissue in Fluorosis rats

In this study, liver of normal rats and *Ocimum* treated rats exhibited normal central vein, hepatocytes and sinusoids. Where as in NaF rats, degeneration of central vein, hepatocytes and sinusoids are observed. However with Ocimum treatment for 60 days regenerated the central vein, hepatocytes and sinusoids in NaF toxicity rats. This study showed the hepatoprotective effect of Ocimum. (Figure 5).

DISCUSSION

The current investigation was carried to know the pharmacological effect *of Ocimum sanctum* phenolic faction in NaF induced fluoosis in rats.

DPPH activity is one of the important free radical scavenging assay which actually depend on the 1,1-diphenyl-2-picrylhydrazyl, which decolorize in presence of antioxidants, such asantioxidant compounds in *Ocimum sanctum*.[20] The antioxidant compounds in *Ocimum sanctum* reacts with DPPH and convert it into1-1-diphenyl-2-picrylhydrazine and this scavenging activity was mainly depends on decolourization potential of methanolic extract of *Ocimum sanctum*. This assay have been used to estimate the free radical scavenging effect of

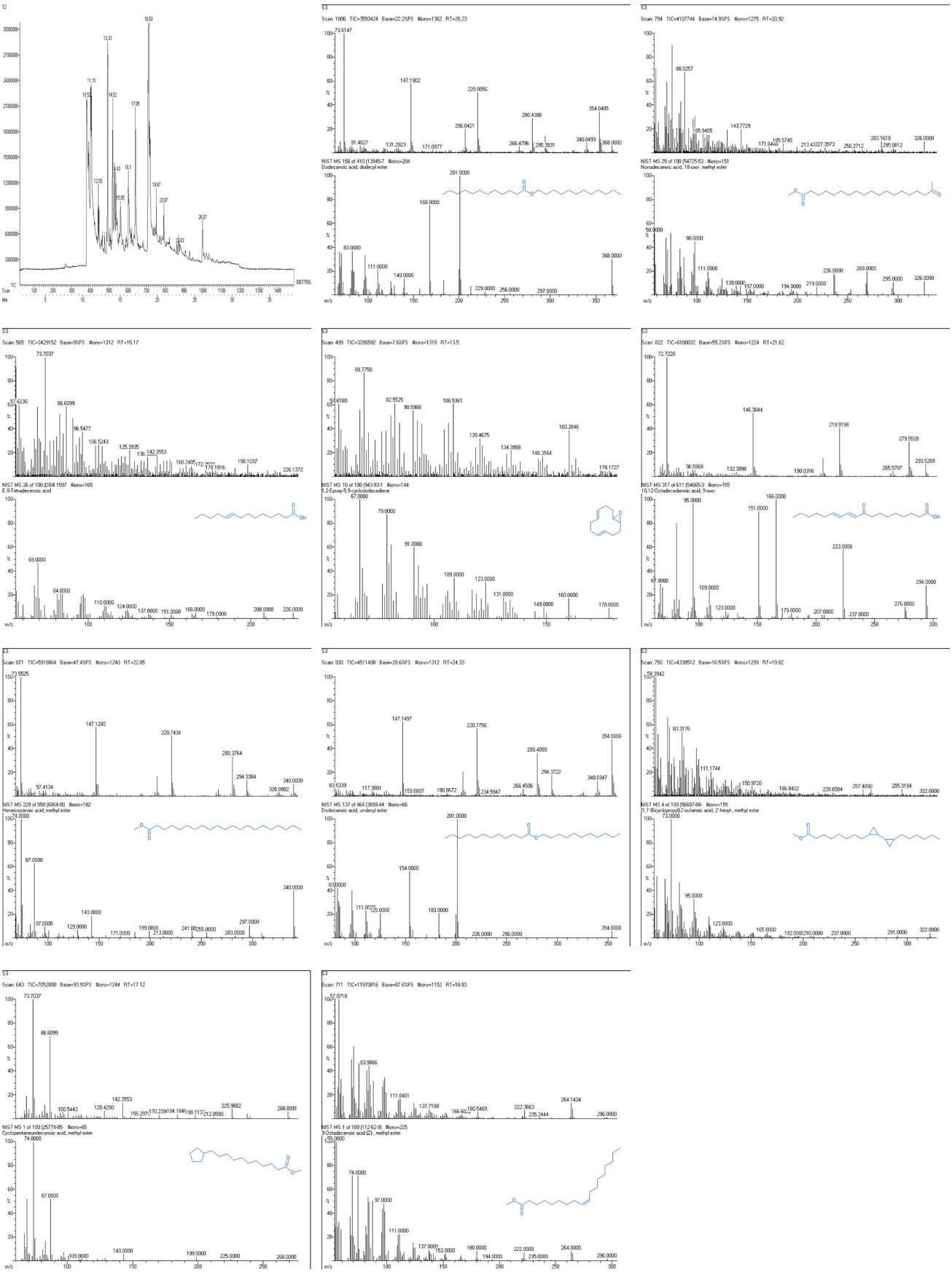

Figure 2: Pictographic Diagram of GC-MS Analysis of *Ocimum sanctum*

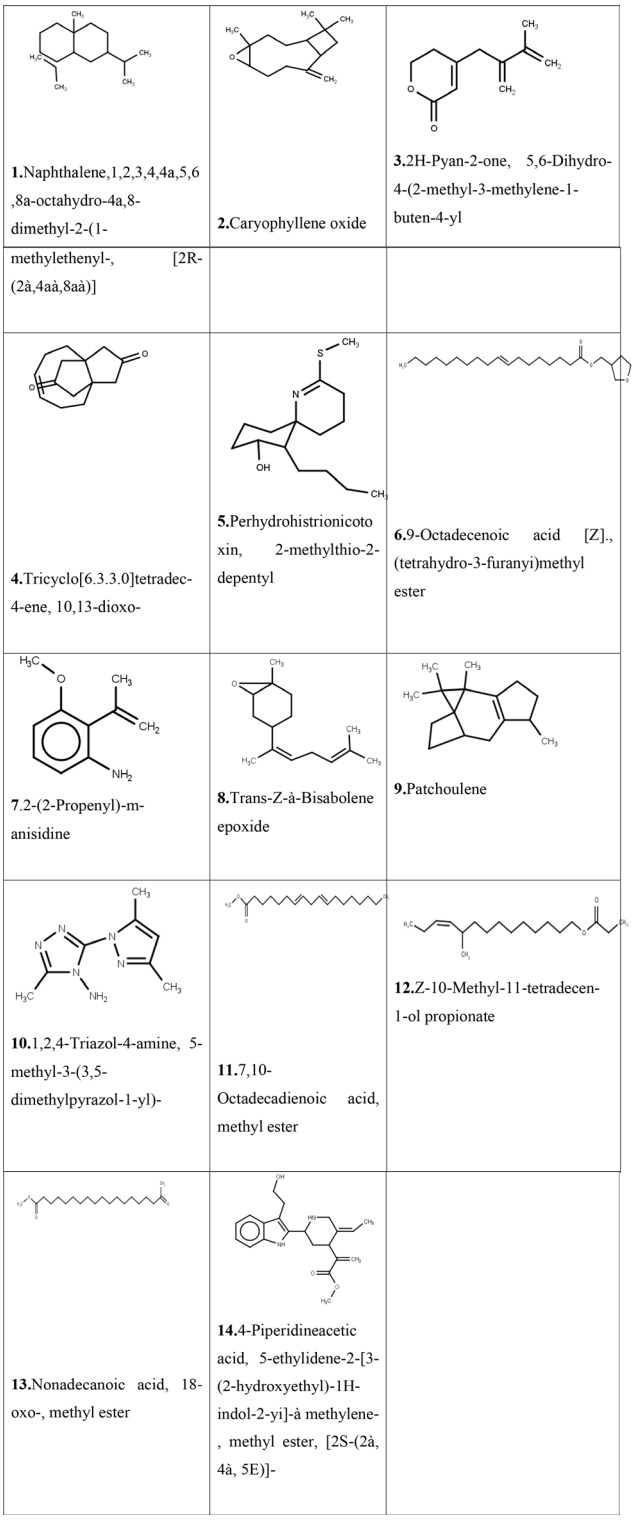

Figure 3: Chemical structures of the Bioactive compounds of *Ocimum sanctum.*

many medicinal plants.[21] Antioxidant activity of *Ocimum sanctum* have been reported by many researchers.[22,23] The standard ascorbic acid exhibits significant DPPH activity than OS methanolic extract.

Table 1: GC-MS Analysis of *Ocimum sanctum* showing the Bioactive compounds and their Molecular weights.

S.NO	Name of the Bioactive Compounds	R TIME	Molecular Weights
1	Naphthalene,1,2,3,4,4a,5,6,8a-octahydro-4a,8-dimethyl-2-(1-methylethenyl-, [2R-(2à,4aà,8aà)]	12.05	238.459
2	Caryophyllene oxide	13.33	220.356
3	2H-Pyan-2-one, 5,6-Dihydro-4-(2-methyl-3-methylene-1-buten-4-yl	11.15	178.231
4	Tricyclo[6.3.3.0]tetradec-4-ene, 10,13-dioxo-	14.02	218.296
5	Perhydrohistrionicotoxin, 2-methylthio-2-depentyl	17.05	269.45
6	9-Octadecenoic acid [Z]., (tetrahydro-3-furanyi)methyl ester	26.08	366.586
7	2-(2-Propenyl)-m-anisidine	10.55	163.220
8	Trans-Z-à-Bisabolene epoxide	14.43	220.356
9	Patchoulene	15.05	216.368
10	1,2,4-Triazol-4-amine, 5-methyl-3-(3,5-dimethylpyrazol-1-yl)-	16.1	192.226
11	7,10-Octadecadienoic acid, methyl ester	18.83	294.479
12	Z-10-Methyl-11-tetradecen-1-ol propionate	19.87	282.468
13	Nonadecanoic acid, 18-oxo-, methyl ester	20.87	326.521
14	4-Piperidineacetic acid, 5-ethylidene-2-[3-(2-hydroxyethyl)-1H-indol-2-yi]-à methylene-, methyl ester, [2S-(2à, 4à, 5E)]-	23.03	354.450

Hydrogen peroxide involves in various important physiological processes of cells.[24] Methanolic extract of *Ocimum sanctum* convert the hydrogen peroxide to water. *Ocimum sanctum* showed potent H_2O_2 scavenging activity at different concentrations.[18] DPPH, H_2O_2 and hydroxyl radical scavenging activity of methanolic extract of *Ocimum sanctum* showed lower effect than standard. The standards i,e ascorbic acid and alpha tocopherol are completely independent compounds hence, they posses good antioxidant effect at different concentrations than methanolic extract of *Ocimum sanctum*. (Figure 1). Free radical scavenging effects are due the presence of phytochemicals and secondary metabolites like phenolic, flavonoids, and other compounds[25] in *Ocimum sanctum*. The importance of free radical scavenging activity is to estimate the antioxidant effect because *Ocimum sanctum* has many antioxidant compounds.[26]

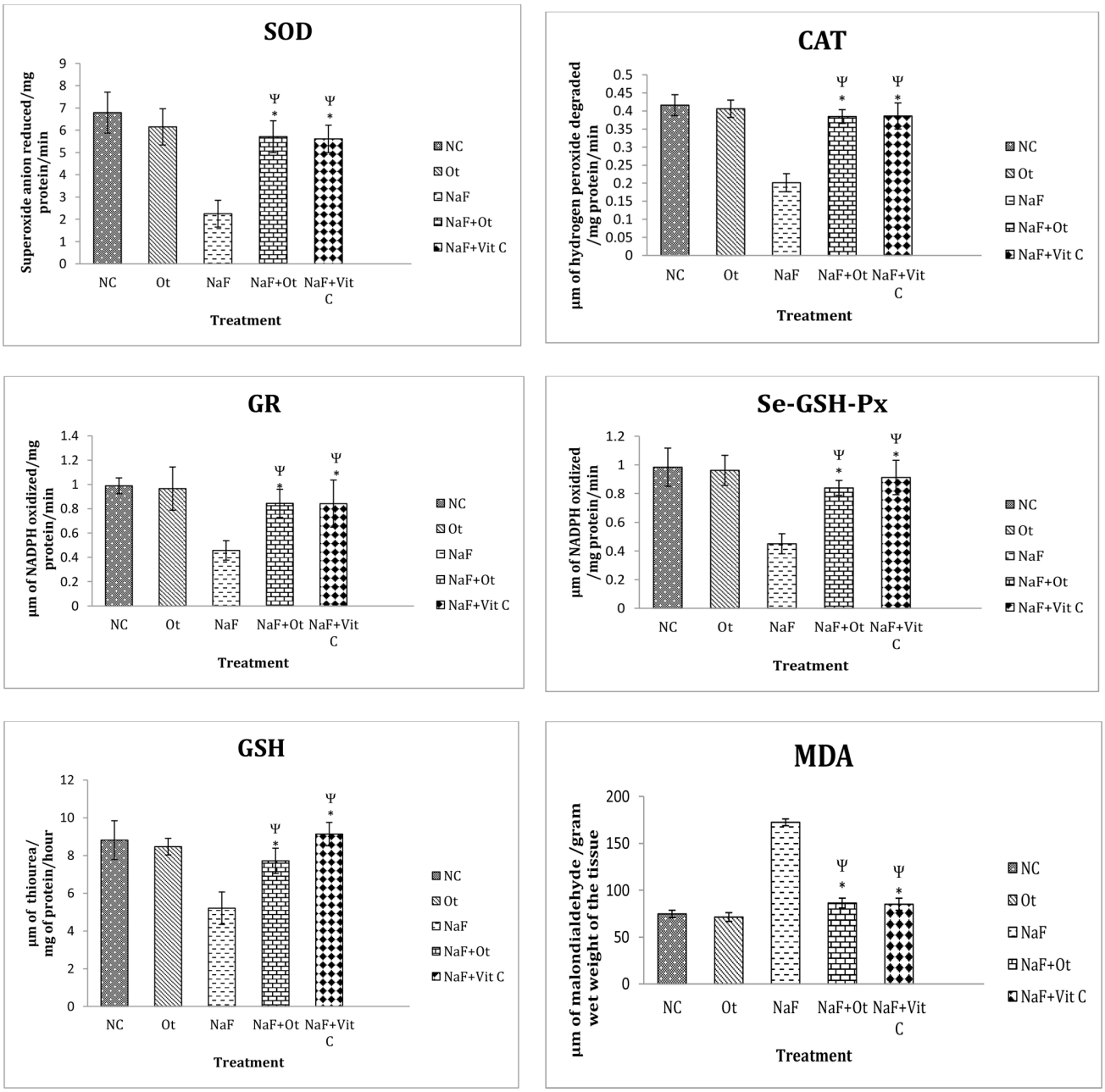

Figure 4: Impact of *Ocimum sanctum* phenolic fraction on SOD, CAT, GR, GPx, activities and GSH, MDA levels in the liver tissues of Normal and Fluorosis rats. Data are expressed as means ± SD (*n* = 6). * The values are significant compared to the following: control (* *p* < 0.01), Fluorosis (p* < 0.01) (Dunnett's multiple comparison tests).**

In our study GC-MS analysis of *Ocimum sanctum* reports many bioactive compounds. These bioactive compounds were recognized through mass spectrum analysis connected with GC. The unknown peaks obtained from the chromatogram are examined with the database of known spectrum of components kept in GC-MS library. The bioactive compounds, which are detected by the GC-MS are shown in Table 1.

In our analysis, we have identified 14 compounds in *Ocimum sanctum* methanolic extract. The compounds are 5-methyl-3-(3,5-dimethylpyrazol-1-yl)-Naphthalene, [2R-(2à,4aà,8aà)],Caryophylleneoxide,5,6-Dihydro-4-(2-methyl-3-methylene-1-buten-4-yl, 9-Octadecenoic acid [Z], (tetrahydro-3-furanyi)methyl ester, 2H-Pyan-2-one, Tricyclo[6.3.3.0] tetradec-4-ene, 10,13-dioxo, Perhydrohistrionicotoxin, 2-methylthio-2-depentyl,Trans-Z-à-Bisabo leneepoxide,2-(2-Propenyl)-m-anisidine, Patchoulene, 1,2,4-Triazol-4-amine, 5-methyl-3-(3,5-dimethylpyrazol-1-yl), 1,2,3,4,4a,5,6,8a-octahydro-4a,8-dimethyl-2-(1-methyl ethenyl-,7,10-Octadecadienoic acid, methyl ester, Z-10-

Table 2: Effect of *Ocimum sanctum* Phenolic fraction on Serum markers in NaF rats.					
Groups	**AST (IU/L)**	**ALT (IU/L)**	**ALP (IU/L)**	**Ca (IU/L)**	**PP (mg/dL**
Group I	586.82	94.12	414	11.31	14.24
(NC)	(±12.48)	(±6.68)	(±8.26)	(±1.69)	(±1.24)
Group II	435	92.535	285	11.82	13.84
(Ot)	(±12.46)	(±10.14)	(±10.42)	(±1.46)	(±1.16)
Group III	606.442*	101.642*	614*	8.2*	16.82*
(NaFt)	(±18.48)	(±8.68)	(±11.28)	(±1.72)	(±1.36)
Group IV	72.267*	67.348*	180*	10.2*	13.26*
(NaF+Ot)	(±4.62)	(±4.52)	(±8.42)	(±1.82)	(±1.32)
Group V	66.731*	51.189*	151*	11.22*	13.86*
(NaF+Vit C)	(±3.14)	(±4.12)	(±6.14)	(±1.12)	(±1.64)

All the values are means ± SD of six individual observations.
* Significant at $p < 0.001$ with respect to normal control.

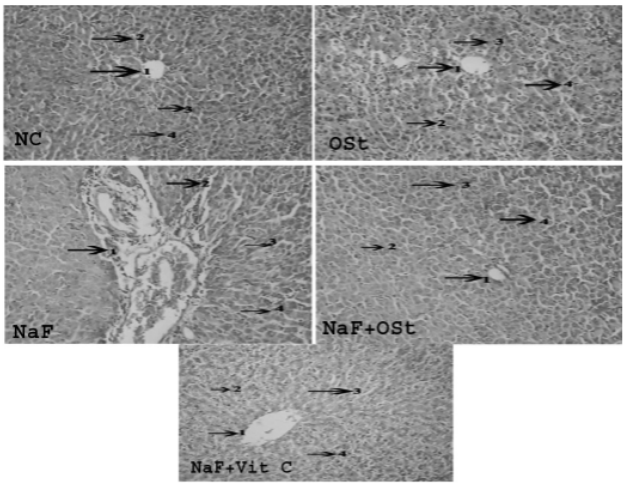

Figure 5: Impact of Ocimum on Liver tissue in NaF intoxicated Fluorosis rats.

1. Normal Control (Nc). 1. Normal central vein, 2. Normal hepatocytes, 3.Normal sinusoids, 4. Normal nucleus. **2. Ocimum treatment (OSt):** 1.Nomal cenntral vein, 2. Normal hepatocytes, 3. Normal sinusoids, 4. Normal nucleus. **3.Sodium fluoride (NaF)** 1. Degeneration of central vein, 2. Degeneration of hepatocytes, 3. Degeneration of sinusoids, 4. Degeneration of nucleus. **4. Sodium fluoride + Ocimum treatment (NaF+OSt)** showed 1. Regeneration of central vein, 2. Regeneration of hepatocytes, 3.Regeneration of sinusoids, 4. Regeneration of nucleus. **5. Sodium fluoride +Vitamin C treatment (NaF+Vit C)** 1. Regeneration of central vein, 2. Regeneration of hepatocytes, 3.Regeneration of sinusoids, 4. Regeneration of nucleus.

Methyl-11-tetradecen-1-ol propionate, Nonadecanoic acid, 18-oxo-, methyl ester, 4-Piperidineacetic acid, 5-ethylidene-2-[3-(2-hydroxyethyl)-1H-indol-2-yi]-à methylene-, methyl ester, [2S-(2à, 4à, 5E)]-These compounds may have pharmacological properties, which are unexplored (Figure 2,3 and Table 1).

In the present investigation, the antioxidant enzymes like SOD, GPx, GR, CAT, GSH are depleted in sodium fluoride induced fluorosis rats. Our study reported that depletion of these antioxidant enzymes in Na F treatment rats. This may be due to the oxidative stress and excess free radicals production in NaF intoxicated rats. However, OS phenolic fraction treatment in NaF rats for 60 days reversed these antioxidant enzymes. Ocimum has many pharmacological properties like antioxidant, hepatoprotective and antimicrobial and other properties. *Ocimum* has many bioactive and pharmacological compounds, these may be responsible for the up regulation of antioxidant enzymes in NaF rats. Ocimum treatment considerably recovered the activities of antioxidant enzymes in NaF intoxicated rats. (Figure 4).

Oxidative stress is reported to be associated with more than 100 diseases like cancer, diabetes and hepatitis. There is a strong relationship between TBA (thiobarbituric acid-reactive substances) and products that reflect oxidative damage to DNA. MDA levels depleted in NaF rats, but with Ocimum treatment reversed back MDA levels, near to normal. This shows the antiperoxidative activity. Hepato toxicity is the condition of oxidative stress in liver generating reactive oxygen species as a result, liver MDA levels increased in NaF rats. However, Ocimum treatment, MDA levels decreased in hepatitis rats. (Figure 4).

In rats, which received NaF for 60 days, showed liver damage and also disturbance of liver cell metabolism which lead to alterations in the activities of many enzymes. Serum enzymes AST, ALP and ALT are markers of liver toxicity and their higher levels are representation of cellular damages and loss of hepatic

cell membrane. In the current study, we reported elevation in AST, ALT and ALP activities in NaF intoxicated rats. Our report showed fluorosis leads to hepatic cellular dysfunction, which results in the upregulation of liver markers. However with Ocimum supplementation these serum markers are depleted in NaF rats. Based on our results, the *Ocimum sanctum* methanolic extract have hepatoprotective potential. OS may reduce the formation of toxic free radicals and reduces the hepatocellular injury in NaF rats (Table 2).

Calcium and Phosphorus are important to humans, because, they help in the formation of bone. In the present study, we observed depleted calcium levels and elevated phosphorus levels in sodium fluoride intoxicated rats. These alterations in calcium and phosphorus levels are due changes in the metabolism of phosphorus and calcium. Whereas Ocimum treatment in NaF rats, for 60 days, these fluorosis markers normalized to near normal levels. (Table 2).

In the current investigation, we reported that in normal control rats, *Ocimum* treated rats the liver architecture is normal. Whereas in fluorosis rat liver, degeneration of central vein, hepatocytes, sinusoids and blood coagulation are observed. But with Ocimum treatment in fluorosis rats, regeneration of central vein, hepatocytes and sinusoids are observed. Histopathological studies also prove that the hepatoprotective effect of Ocimum in NaF rats. (Figure 5).

CONCLUSION

The current study proved that phenolic fraction of *Ocimum sanctum* protects the hepatic tissue from sodium fluoride intoxication in albino rats. We also reported that antioxidant enzymes, hepatic markers and flurosis markers are normalized with *Ocimum* treatment in Fluorosis rats. GC-MS analysis reported many bioactive compounds in *Ocimum sanctum* and these compounds may responsible for anti-fluorosis effect and antioxidant effects. This is the first study on the effect of phenolic fraction of *Ocimum sanctum* in fluorosis rats.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ABBREVIATIONS

OS: *Ocimum sanctum*; **SOD:** Superoxide dismutase; **GPx:** Glutathione peroxidase; **GSH:** Glutathione; **GR:** Glutathione reductase; **GST:** Glutathione-s-transferase; **CAT:** Catalase; **MDA:** Malondialdehyde; **AAT:** Alanine aminotransferase; **ALKP:** Alkaline phosphatase; **AST:** Aspartate aminotransferase; **P4:** Phosphorus; **Ca:** Calcium; **DPPH:** 1,1-diphenyl-2-picryl hydrazyl radical; **H_2O_2:** Hydrogen peroxide; **GC-MS:** Gas chromatography–mass spectrometry; **MS:** Mass Spectroscopy; **NIST:** National Institute of Standard and Technology; **TBA:** thiobarbituric acid-reactive substances.

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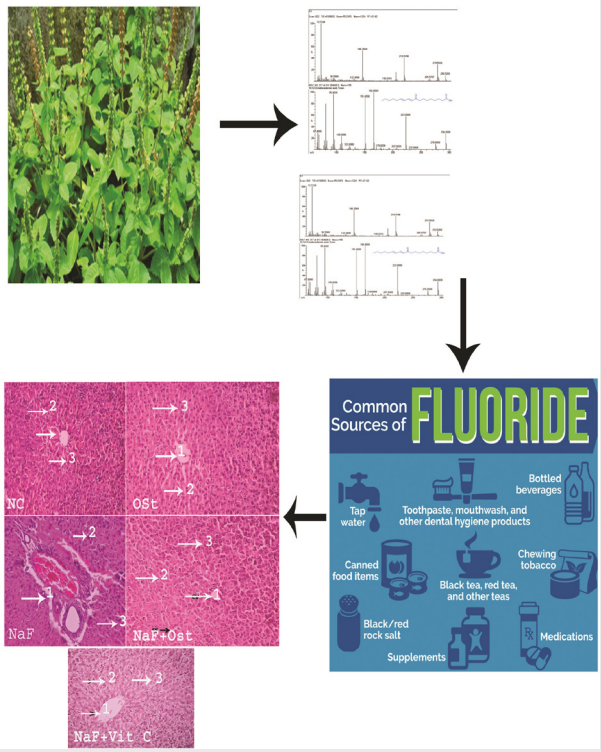
PICTORIAL ABSTRACT



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