Preliminary Study of Antioxidant Potential and Gas Chromatography-mass Spectroscopy (GC-MS) Analysis of \textit{Brassica oleracea} Florets

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Abstract

Introduction: \textit{Brassica oleracea var. Italica} ordinarily known as “broccoli” is a cruciferous green leafy vegetable and has been reported to have antioxidant, anti-inflammatory, antibacterial, anticancer, antiatherosclerotic, antimucolytic, antinitrosaminic, anti-nyctalopic, antiproliferant, and hypocholesterolemic potential. To determine the presence of bioactives, preliminary phytochemical screening of methanol extract was done and its antioxidant potential was evaluated by \textit{in vitro} radical scavenging assays. Phytoconstituents present in the extract were identified by gas chromatography-mass spectroscopy (GC-MS) analysis. Materials and Methods: In the preliminary phytochemical screening, various chemical tests were performed. Evaluation of the \textit{in vitro} antioxidant efficacy of the extract was done by 1,1-Diphenyl-2-picrylhydrazyl (DPPH) scavenging assay and ferrous ion-chelating ability assay. Various phytoconstituents were identified on the basis of retention indices and mass spectra fragmentation pattern obtained through GC-MS studies. Results: Phytochemical screening of the floret extract revealed the presence of terpenoids, alkaloids, tannins, steroids, and saponins. Plant has significant scavenging effect on DPPH (half maximal inhibitory concentration [IC$_{50}$] = 0.070 ± 0.67 mg/ml) which is comparable to that of ascorbic acid (IC$_{50}$ = 0.045 ± 0.61 mg/ml) and butylated hydroxytoluene (BHT) (IC$_{50}$ = 0.118 ± 0.53 mg/ml). The highest chelating activity of methanol extract and ethylenediaminetetraacetic acid is found to be 83.69% and 98.91%, respectively. GC-MS analysis report confirmed the presence of ascorbic acid 2,6-dihexadecanoate, phytosterols, linoleic acid, palmitic acid, and oleic acid as the major constituents. Terpenoids, namely squalene and geraneol, were found in traces. Discussion and Conclusion: From the study, it has been concluded that the \textit{B. oleracea} is a potential source of plant-based therapeutics and a natural source of antioxidants. It has been found that the extract contains a significant amount of phytochemicals with antioxidants which could act as scavengers of free radicals. From this study, we got a sound base for further investigation of \textit{B. oleracea} for its pharmaceutical application.

Key words: 1,1-Diphenyl-2-picrylhydrazyl, antioxidant, fluorescence recovery after photobleaching, gas chromatography-mass spectroscopy, phytochemical screening

INTRODUCTION

Plant plays a vital role for the healthfulness of the human being and is the major source of medicinally important compounds. Since the ancient time, people all around the world are using indigenous flora for medicinal purpose. \textit{Brassica oleracea} or broccoli is one of the prominent edible herbs and has been categorized as functional food as it contains normal dietary constituents and has plenty of therapeutically active components as well which are helpful in delaying or preventing a large number of ailments.\textsuperscript{[1]} \textit{B. oleracea} has health-promoting properties such as antioxidant, anticarcinogenic, anti-atherosclerotic, antibacterial, antimaculitic, anti-nitrosaminic, anti-nyctalopic, antiproliferant, antioxidant, anti-radicular, anti-retinitic, antitumor, antiviral,
detoxicant, estrogenic, glucuronidase inhibitor, goitrogenic, hypcholesterolemic, prooxidant, quinone-reductase-inducer, and anticonvulsant.\textsuperscript{[2,3]} It is mainly composed of polyphenols, glucosinolates, sulforaphane, and selenium.\textsuperscript{[4]}

Broccoli is a low-calorie vegetable; it provides just 34 calories per 100 g.\textsuperscript{[5,6]} Broccoli have plethora of medicinally important compounds such as alkaloids, tannins, flavonoids, and phenols which have antioxidants, antibacterial, anti-inflammatory, anticancer, and hepatoprotective potential. Antioxidants are the active compounds which have the capability of neutralizing the free radicals present inside the human body. Free radicals are the reactive species produced in metabolic process and by air pollution, smoking, and radiation in human body. These free radicals react with various cell organelles may cause cell death or chronic diseases such as cardiovascular dysfunctions, neurodegeneration, aging, and weakening of immune system.\textsuperscript{[7]} Chemical compounds present in the plant as secondary metabolites and have various structural arrangements. For the therapeutic knowledge of the reported compounds and also for the exploration of new compounds, analysis of chemical components of her bios must be done. Preliminary phytochemical screening is quite useful in the detection of the bioactive principles. To further carry out drug discovery and spectroscopic studies, mass spectrometry (MS), coupled with chromatographic separations technique like gas chromatography-MS (GC-MS), is a very good technique.\textsuperscript{[8]} On the basis of above facts, the current study was planned to perform phytochemical screening and evaluate antioxidant potential of using various assays such as 1,1-Diphenyl-2-picrylhydrazyl (DPPH) and fluorescence recovery after photobleaching (FRAP). Besides, identification of phytoconstituents was done by GC-MS analysis taking the retention indices (RIs) and mass fragmentation pattern of each compound into consideration. RIs or Kováts index of an analyte is its relative time position between the nearest n-alkanes which elute immediately before and after a target analyte. In the GC-MS, retention time (Rt) is the prime variable considered, but it is a function of experimental conditions and has low reproducibility. Moreover, for the identification of branched alkyl substituents, mass spectral differences are not significant. Combination of retention data with MS provides accurate identification of the compounds. Comparing the known RIs from a retention-data library with measured values is the standard approach to identification.\textsuperscript{[9]}

\section*{MATERIALS AND METHODS}

\subsection*{Collection and identification of plant material}

Florets of \textit{B. oleracea} were collected from open fields, Sikanderpur, Sirsa, Haryana, in December 2016 and identified by Dr. Anjula Pandey, Principal Scientist, ICAR- National Bureau of Plant Genetic Resources, Pusa Campus New Delhi, vide reference no. NHCP/NBPGR/2017-23. The plant was identified as \textit{B. oleracea} var. \textit{italica} Plenck, (Brassicaceae). A voucher specimen is retained in the Department of Pharmaceutical Sciences, Guru Jambeshwar University of Sciences and Technology, Hisar, for future reference. Florets were used to carry out the experimental work procedures pertaining to phytochemical and \textit{in vitro} antioxidant evaluations.

\subsection*{Preparation of extracts}

500 g of air-dried crude powder of florets of \textit{B. oleracea} was defatted first with petroleum ether (60°C–80°C) for 7 days by cold maceration. The defatted drug was then extracted with methanol (95%) as solvent by continuous hot percolation in Soxhlet apparatus for 72 h. Solvent was removed using rotary vacuum evaporator and a semi-solid mass was obtained which is stored in desiccators for further use.

\subsection*{Preliminary phytochemical screening}

Methanol extract of \textit{B. oleracea} was subjected to phytochemical tests for terpenoids, alkaloids, tannins, steroids, and saponins.\textsuperscript{[10,14]}

\subsection*{In vitro antioxidant assays}

\subsection*{DPPH radical scavenging assay}

Antioxidant potential of extract was estimated by determining its DPPH radical scavenging capability by adopting the method of Roy \textit{et al.}\textsuperscript{[15]} with minor modifications. The dried methanol extract was diluted from 0.02 mg/ml to 0.12 mg/ ml. 1 ml of 0.135 mM DPPH solution (in methanol) was added to 1.0 ml of extract (in methanol). This mixture was vortex mixed and incubated in the dark for 30 m at room temperature. Scavenging of DPPH radical by the extract solution converts the DPPH solution from violet to orange, yellow or pale yellow color. Absorbance of the mixture was measured at 517 nm. Ascorbic acid and butylated hydroxyl toluene (BHT) were taken as standards. Scavenging of DPPH radicals by the extract was calculated using the following formula:

\begin{equation}
\%\, inhibition = \left(\frac{\text{Abs of control} - \text{Abs of sample}}{\text{Abs of control}}\right) \times 100
\end{equation}

Where Abs of control is the absorbance of DPPH only.

Abs of sample is the absorbance of the DPPH radical + sample extract/standard.

\subsection*{Metal ion chelating ability assay}

The chelating ability of ferrous ion by the plant extract was estimated by adopting the method followed by Rajauria \textit{et al.}\textsuperscript{[16]} with some modifications. In this assay,
blue-colored ferrous ion-ferrozine complex is formed which has a maximum absorbance at 562 nm. In the assay procedure, 100 µl of different concentrations of extract samples/standard was mixed with 100 µl of deionized water. 25 µl of ferrous chloride (0.5 mm) was added to initiate the reaction. Then, the mixture was shaken vigorously and incubated at ambient temperature for 10 min. Absorbance was recorded at 562 nm. Ethylenediaminetetraacetic acid (EDTA) was used as a standard. The percentage of inhibition of ferrozine–Fe2+ complex formation was calculated using equation (1).

**RESULTS**

**Preliminary phytochemical screening**

Chemical tests have shown positive results for terpenoids, alkaloids, tannins, steroids, and saponins.

**In vitro antioxidant potential**

**DPHH radical scavenging assay**

The DPPH scavenging assay has been widely used to assess antioxidant properties. The concentration-response curve of DPPH radical scavenging activity of the plant extracts, BHT, and ascorbic acid is shown in Figure 1. The flower extract has shown more scavenging than BHT; however, ascorbic acid is more active than the extract. Ascorbic acid has highest inhibition (93%), as compared to that of extract (73%) and BHT (55%). The half maximal inhibitory concentration (IC$_{50}$) values (the IC$_{50}$ denoted the concentration of sample required to scavenge 50% of DPPH free radicals.) obtained for extract, ascorbic acid, and BHT were 0.070 ± 0.67 mg/ml, 0.045 ± 0.61 mg/ml, and 0.118 ± 0.53 mg/ml, respectively [Table 1].

**Metal ion chelating ability assay**

Metal ion chelating efficacy of the plant extract is comparable with that of EDTA (used as standard). Plant extract has shown 83.69% inhibition, and EDTA has shown 98.91 % inhibition at 0.12 mg/ml concentration. IC$_{50}$ of extract is 0.061±0.32 and that of EDTA is 0.056.69 ± 0.32 [Table 1 and Figure 2].

**GC-MS analysis**

GC-MS analysis of *B. oleracea* extract resulted in the identification of 25 compounds [Table 2]. Figure 3 shows GC-MS chromatogram of floret extract. Major phytoconstituent identified was hexadecanoic acid also called as ascorbic acid 2,6 dihexa decanoate. Many fatty acids along with their esters have also been identified such as heptadecanoic acid, octadecadienoic acid, tetradecanoic acid.
acid glyceryl 2 linoleate, and (Z,z)-6,9-cis-3,4-epoxy
nonadecadiene its esters. Furthermore, some phytosterols
such as stigmasterol and gamma-sitosterol were present in
significant quantity. In addition to all above compounds,
some other compounds were also present which includes
Vitamin E, 2-Hydroxy-1-(1’-pyrrolidiyl)-1-buten-3-one,
diphenylmethanone, pentadecafluorooctanoic acid, and
dodecyl ester. Some flavonoids, namely 2,4,5-trimethyl-
1,3-dioxolane 2,5-Dimethyl-2,4-dihydroxy-3(2H)-furanon,
has also been reported. Broccoli also contains phenolic
compound viz. 4-vinylguaia, amino acid L-pyroglutamic
acid, a sulphur compound-dimethoxysulfone and a
glycoside namely 2,4-dihydroxy-2,5-dimethyl-3(2H)-furan-
3-one was also found in the extract. Two lactones, namely,
4-cyclobutanoic acid and 1,2-cyclopentadiene were also
present in the extract. One diterpene alcohol 2-hexadecen-
1-ol, 3,7,11,15-tetramethane and two triterpenes Stigmasta-
5,22-dien-3-ol, acetate, (3.beta.) and Ergost-5-en-3-ol,
(3.beta.,24r)- were also present.

**DISCUSSION**

Herbs play a vital role as antioxidant against the free radicals
formed in various biological processes inside the human
body. These antioxidants combine with the reactive oxygen
species and terminate the chain reaction before the vital
cellular components are damaged. The principal secondary
metabolites such as flavonoids, polyphenols, tannins, and
saponins are responsible for the antioxidant property of
herbs. To ascertain the antioxidant potential of *B. oleracea*,
these secondary metabolites have been studied qualitatively
as well as quantitatively and also there antioxidant potential
was assessed by *in vitro* antioxidant assays. The preliminary
phytochemical study confirmed the presence of terpenoids,
alcaloids, tannins, steroids, and saponins in the methanol
extract of *B. oleracea*. The GC-MS analysis of *B. oleracea*
flower extract revealed the presence of 25 compounds
having many therapeutic effects. As per the GC-MS results,
*B. oleracea* has many antioxidant compounds likewise
4-vinylguaia (0.88%), myristic acid and ethyl ester (1.29),
ascorbic acid 2,6-dihexadecanoate (14.68%), heptadecanoic
acid (0.70 %), Vitamin E (0.56), stigmasterol (0.74%), and
hexadecanoic acid and 2-hydroxy-1-(hydroxymethyl) ethyl
ester (1.09%). Stigmasterol an unsaturated phytosterol
is a precursor of progesterone, which is helpful in tissue
rebuilding mechanisms related to estrogen effects, and also
acts as an precursor of Vitamin D3 and intermediate in the
biosynthesis of androgens, estrogens, and corticoids.[34] Plant
also has anti-inflammatory compounds such as 4H-Pyran-
4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl (5.46%), and
2-methoxy-4-vinylphenol (0.88%) and few antitumor agents
such as delta (2)-angelica lactone (0.42%), tetradecanoic
acid and ethyl ester (1.29%), octadecenoic acid and ethyl

<table>
<thead>
<tr>
<th>Samples</th>
<th>Extract (IC₅₀ in mg/ml)</th>
<th>Ascorbic acid (IC₅₀ in mg/ml)</th>
<th>BHT (IC₅₀ in mg/ml)</th>
<th>EDTA (IC₅₀ in mg/ml)</th>
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<tbody>
<tr>
<td>DPPH</td>
<td>0.070±0.67</td>
<td>0.045±0.61</td>
<td>0.118±0.53</td>
<td>-</td>
</tr>
<tr>
<td>FRAP</td>
<td>0.061.09±0.53</td>
<td>-</td>
<td>-</td>
<td>0.056.69±0.32</td>
</tr>
</tbody>
</table>

BHT: Butylated hydroxytoluene, EDTA: Ethylenediaminetetraacetic acid, DPPH: 1,1-Diphenyl-2-picrylhydrazyl, FRAP: Fluorescence recovery after photobleaching, IC₅₀: Half maximal inhibitory concentration
Table 2: Phytoconstituents identified along with their RIs, nature, and pharmacological action

<table>
<thead>
<tr>
<th>Rt</th>
<th>RI</th>
<th>Percentage area</th>
<th>Name</th>
<th>Synonyms</th>
<th>Molecular formula</th>
<th>Molecular weight</th>
<th>Nature of compound</th>
<th>Pharmacological action</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.712</td>
<td>917</td>
<td>0.34</td>
<td>4-Cyclobutanoic acid</td>
<td>Dihydro-2 (3 h)-furanoan</td>
<td>C4H7ClO2</td>
<td>122</td>
<td>Lactone</td>
<td>Anti-inflammatory activity, analgesic, ulcerogenic activity</td>
<td>[17]</td>
</tr>
<tr>
<td>4.946</td>
<td>929</td>
<td>0.42</td>
<td>1,2-cyclopentanedione</td>
<td>Delta,(2)-angelica lactone</td>
<td>C5H6O2</td>
<td>98</td>
<td>Carbohydrate</td>
<td>Prevented gastrointestinal tumor growth</td>
<td>[18]</td>
</tr>
<tr>
<td>5.957</td>
<td>984</td>
<td>0.48</td>
<td>2,4-Dihydroxy-2,5-dimethyl-3 (2H)-furan-3-one</td>
<td>pyrone</td>
<td>C6H8O4</td>
<td>144</td>
<td>Glycoside</td>
<td>Antifungal and anti-insectant</td>
<td>[19]</td>
</tr>
<tr>
<td>7.476</td>
<td>1063</td>
<td>0.51</td>
<td>S-Methyl methanethiosulphonate</td>
<td>Dimethoxysulfone</td>
<td>C2H6O2S2</td>
<td>126</td>
<td>Organosulfur</td>
<td>Suppresses chromosome aberrations, antimutagenic agent</td>
<td>[20]</td>
</tr>
<tr>
<td>9.179</td>
<td>1151</td>
<td>5.46</td>
<td>4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl</td>
<td>2,4,5-Trimethyl-1,3-dioxolane</td>
<td>C6H8O4</td>
<td>144</td>
<td>Flavonoid fraction</td>
<td>Anti-inflammatory, analgesic, antibacterial, antifungal</td>
<td>[21]</td>
</tr>
<tr>
<td>12.160</td>
<td>1314</td>
<td>0.88</td>
<td>2-methoxy-4-vinylphenol</td>
<td>4-vinylguaia Col</td>
<td>C9H10O2</td>
<td>150</td>
<td>Phenolic compound</td>
<td>Antioxidant antimicrobial anti-inflammatory</td>
<td>[22]</td>
</tr>
<tr>
<td>13.366</td>
<td>1385</td>
<td>0.89</td>
<td>DL-Proline, 5-oxo-, methyl ester</td>
<td>L-Pyroglutamic acid</td>
<td>C6H9NO3</td>
<td>143</td>
<td>Natural amino acid</td>
<td>For mental fatigue and memory improvement</td>
<td>[23]</td>
</tr>
<tr>
<td>15.471</td>
<td>1515</td>
<td>1.71</td>
<td>2-Hydroxy-1-(1'-pyrrolidyl)-1-buten-3-one</td>
<td>Menthone-d1</td>
<td>C8H13NO2</td>
<td>155</td>
<td>Alcohol</td>
<td>Antiseptic, anesthetic, local pain, pruritis, allergic dermatitis</td>
<td>[24]</td>
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<td>17.274</td>
<td>1637</td>
<td>2.78</td>
<td>Diphenylmethane</td>
<td>Trioxolane</td>
<td>C13H10O</td>
<td>182</td>
<td>Ozonoides</td>
<td>Antiviral and antimicrobial</td>
<td>[25]</td>
</tr>
<tr>
<td>17.868</td>
<td>1678</td>
<td>1.72</td>
<td>Pentadecafluoroctanoic acid, dodecyl ester</td>
<td>Cetylpyridinium chloride</td>
<td>C20H25F15O2</td>
<td>582</td>
<td>Cationic quaternary ammonium compound</td>
<td>Antibacterial</td>
<td>[26]</td>
</tr>
<tr>
<td>19.027</td>
<td>1762</td>
<td>1.29</td>
<td>Tetradecanoic acid, ethyl ester</td>
<td>Myristic acid, ethyl ester</td>
<td>C14H28O2</td>
<td>228</td>
<td>Fatty acid</td>
<td>Antioxidant, cancer preventive</td>
<td>[27]</td>
</tr>
<tr>
<td>20.762</td>
<td>1893</td>
<td>1.00</td>
<td>Octadecanoic acid, ethyl ester</td>
<td>Ethyl oleate</td>
<td>C14H22N2O</td>
<td>234</td>
<td>Fatty acid ester</td>
<td>Hemolytic agent cancer preventive</td>
<td>[27]</td>
</tr>
<tr>
<td>21.691</td>
<td>1968</td>
<td>14.68</td>
<td>Hexadecanoic acid, methyl ester</td>
<td>Ascorbic acid, 2,6-dihexadecanoate</td>
<td>C16H32O2</td>
<td>256</td>
<td>Palmitic acid ester</td>
<td>Antioxidant</td>
<td>[28]</td>
</tr>
<tr>
<td>22.487</td>
<td>2034</td>
<td>0.70</td>
<td>Heptadecanoic acid</td>
<td>Ethyl margarate</td>
<td>C17H34O2</td>
<td>270</td>
<td>Fatty acid</td>
<td>Antioxidant</td>
<td>[27]</td>
</tr>
<tr>
<td>Rt</td>
<td>RI</td>
<td>Percentage area</td>
<td>Name</td>
<td>Synonyms</td>
<td>Molecular formula</td>
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<td>Nature of compound</td>
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<td>Reference</td>
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<tr>
<td>23.782</td>
<td>2145</td>
<td>15.39</td>
<td>(Z, z)-6,9-cis-3, 4-epoxy-nonadecadiene</td>
<td>Linoleic acid chloride</td>
<td>C19H34O</td>
<td>278</td>
<td>-</td>
<td>No activity reported</td>
<td>[29]</td>
</tr>
<tr>
<td>24.018</td>
<td>2165</td>
<td>2.66</td>
<td>Octadecanoic acid</td>
<td>Stearic acid</td>
<td>C18H36O2</td>
<td>284</td>
<td>Polyenoic fatty acid</td>
<td>Decreases plasma cholesterol</td>
<td>[30]</td>
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<tr>
<td>28.067</td>
<td>2508</td>
<td>1.09</td>
<td>Hexadecanoic acid, 2-hydroxy-1- 2-Monopalmitoylglycerol</td>
<td>(hydroxymethyl) ethyl ester</td>
<td>C19H38O4</td>
<td>330</td>
<td>Amino compound</td>
<td>Hemolytic, pesticide, flavor, antioxidant</td>
<td>[31]</td>
</tr>
<tr>
<td>30.021</td>
<td>2599</td>
<td>3.49</td>
<td>Nonanoic acid, 9-(3-hexenylidenecyclopropylidene</td>
<td>Glyceryl2 linoleate</td>
<td>C21H36O4</td>
<td>352</td>
<td>Fatty acid ester</td>
<td>Cosmetic, coloring agent</td>
<td>[31]</td>
</tr>
<tr>
<td>33.026</td>
<td>3138</td>
<td>0.83</td>
<td>Stigmasta-5,22-dien-3-ol, acetate, (3.beta.)</td>
<td>Stigmasterol acetate</td>
<td>C31H50O2</td>
<td>454</td>
<td>Triterpene</td>
<td>Antihepatotoxic, anti-inflammatory, antiphilic, antioxidant, artemicidal, extrogenic, sedative</td>
<td>[15]</td>
</tr>
<tr>
<td>33.230</td>
<td>3149</td>
<td>0.92</td>
<td>1,2-hexadecanediol</td>
<td>ARACHIDIC alcohol</td>
<td>C16H34O2</td>
<td>258</td>
<td>Alcohol compound</td>
<td>Nf</td>
<td>-</td>
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<tr>
<td>33.490</td>
<td>3163</td>
<td>2366773</td>
<td>Vitamin E</td>
<td>Alpha.-tocopherol</td>
<td>C29H50O2</td>
<td>430</td>
<td>Organic compound</td>
<td>Antiaging, analgesic, antiobiotic, anti-inflammatory, antioxidant, antidermatitic, antiulcerogenic, vasodilator, antispasmodic, antibronchitic, anticonorany</td>
<td>[21]</td>
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<tr>
<td>34.620</td>
<td>3240</td>
<td>3.16</td>
<td>Ergost-5-en-3-ol, (3.beta.,24r)-</td>
<td>Stigmasterol acetate</td>
<td>C28H48O</td>
<td>400</td>
<td>Phytosterol</td>
<td>Dyslipidemia and cardiovascular</td>
<td>[32]</td>
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(Contd...)
ester (1.00%), and Vitamin E (0.56%). Some antifungal, antibacterial, and antiviral compounds have also been found, namely 2,4-Dihydroxy-2,5-dimethyl-3(2H)-furan-3-one (0.48%), 2-Hydroxy-1-(1'-pyrrolidinyl)-1-buten-3-one (1.71%), trioxolane (2.78%), pentadecafluorooctanoic acid, and dodecyl ester (1.72%). Few antidiabetic/hypoglycemic compounds have also been reported such as Vitamin E (0.56%), Stigmasterol (0.74%), Gamma-sitosterol (12.45%), hypcholesterogenic agent ergost-5-en-3-ol, (3.beta., 24r)- (3.16%), octadecanoic acid (2.66%), and an organosulfur compound dimethoxysulfone (0.51%) having antimutagenic property which is also present in the extract. Plant extract also has a natural amino acid, namely L-pyroglutamic acid, which has been reported to be useful in memory improvement and mental health.

CONCLUSION

The present study demonstrates that methanol extract of *B. oleracea* has a significant antioxidant potential. Furthermore, it has many therapeutically active constituents which can be of great importance for the pharmaceutical industry. These compounds should be isolated and explored further for their medicinal use.

ACKNOWLEDGMENT

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