

## Spectrophotometric assay of Anti-oxidative and Free Radical Scavenging activities of *Crataeva nurvala* leaf extract

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**Abstract:** The study aimed to evaluate the anti-oxidative activity of *Crataeva nurvala* leaf polyphenols by estimation of total phenolics extracted in different solvents under Microwave Assisted Extraction system and by evaluation of its adical scavenging and lipid per-oxidation inhibition properties. Total polyphenols were quantitatively estimated in solvents of Gr-A (100% methanol), Gr-B (100% de-ionised water), Gr-C (methanol: water: acetic acid in 70:25:05 v/v), Gr-D (ethyl acetate: methanol: water in 60:30:10 v/v), Gr-E (100% acetone), Gr-F (acetone: water: acetic acid in 90: 9.5: 0.5 v/v) and Gr-G (100% ethanol). Since, organic and aqueous solvent mixture groups of Gr-C, D and F exhibited significantly ( $p<0.01$ ) higher concentration of total polyphenols, these were used for assay of total and individual anti-oxidative activities. Significantly higher ( $p<0.01$ ) total polyphenols were observed in solvent mixtures of methanol with other solvents at Gr-D and C and in acetone at Gr-F as compared to other individual solvents at Gr-A, B, E and G. The polyphenols in Gr-D exhibited significantly higher anti-oxidant activities ( $p<0.01$ ) in phosphor-molybdenum and reducing power method than the solvents at Gr-C and F. Bu, the polyphenols at Gr-C solvent observed significantly higher activities ( $p<0.01$ ) in FRAP method followed by Gr-D and F. The Gr-D solvent pose significantly higher SO and NO scavenging activities ( $p<0.01$ ) followed by solvents of Gr-C and F whereas, OH scavenging activity and LPOI is significantly ( $p<0.01$ ) higher in Gr-F solvents followed by that of Gr-C and D. *Crataeva nurvala* leaf is a rich source of polyphenols which can be better recovered in mixtures of organic and aqueous mixture. Its medicinal value is attributed to the potent anti-oxidative effect against *in vitro* chemical induced free radical generation and lipid per-oxidation.

**Keywords:** *Crataeva nurvala*, Total phenolics, Anti-oxidant, FRAP.

### INTRODUCTION:

Oxidative stress of endogenous / exogenous origin can be overcome by chain-breaking antioxidants (CBAs) as 1<sup>st</sup> line of defense like tocopherol, ascorbic acid, glutathione (GSH), uric acid, carotenoids, ubiquinone, and polyphenols. The ongoing reaction can be terminated / interrupted by autocatalytic reactions of antioxidant enzymes viz superoxide dismutase (SOD), catalase (CAT), and GPxs as 2<sup>nd</sup> line of defense. With advancement of age and decline of anti-oxidative defense system, natural and synthetic anti-oxidants are essential. Constraints of producing adverse effects on liver restrict the use of synthetic antioxidants butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT). Natural antioxidants of plant origin has been associated with reduced risks of cancer, cardiovascular disease, diabetes, inflammation, bacterial disease,

diseases associated with ageing<sup>1,2</sup> owing to their antioxidant and free radical scavenging activities<sup>3</sup> by blocking the initiation or propagation of oxidizing chain reactions or by scavenging various types of reactive species or chelating transition metal ions<sup>4</sup>. Since, lead molecules of plant origin are safer, cheaper, locally available, easily consumable (raw) and simple it serve as an alternative medicine<sup>5</sup>. Even though, the efficacy of phyto-chemicals is known to the scientific world but mechanisms of their systematic action is less known<sup>6</sup>. Phyto-components vary in chemical characteristics, polarities and distribution in the plant matrix<sup>7</sup>. Therefore, extraction conditions viz nature of solvent and technique used to harvest the bio-active components determine their efficacy/potency<sup>8</sup>.

The claim of medicinal plants for posing anti-oxidant, anti-inflammatory, anti-tumor, anti-mutagenic, anti-carcinogenic, anti-bacterial, anti-viral and hepato-protective activities are mainly attributed to polyphenols. Polyphenols are the secondary plant metabolites with one / more phenolic hydroxyl groups attached to carbon-based aromatic phenyl-ring. There are over 8,000 structural variants of polyphenols distributed variedly in the plant kingdom. *Crataeva nurvala* of Family Capparidaceae is a medium sized branched deciduous indigenous plant of India<sup>9</sup>. Tribes of Orissa province traditionally use aqueous paste of this plant with ghee and honey for treatment of rheumatoid arthritis, hepatic disorders, splenomegally, fever, anorexia, obesity, diabetes, body ache, itches, wounds, stomach-ache, flatulent dyspepsia, helminthiasis, diarrhoea, dysentery, urinary calculi, dysuria and cystitis. The crude plant extract pose anti-fertility<sup>10</sup>, analgesic, anti-diarrhoeal<sup>11</sup>, anti-arthritic<sup>12</sup>, cardio-protective<sup>13</sup>, hepato-protective<sup>14</sup>, urolithic<sup>15</sup>, anti-nociceptive<sup>16</sup>, anti-diabetic<sup>17</sup>, anti-inflammatory<sup>18</sup> and anti-cancer<sup>19</sup> activities. So, it has been incorporated in Ayurvedic preparations of Varunadi quath, Varunadya ghrta and Varunadya taila<sup>20</sup>. Therefore, the present study aims to recover the polyphenols from *Crataeva nurvala* leaves in different solvents and to assay the in vitro anti-oxidative effect through various chemical tests for advocating its efficacy on validation and development of lead molecules.

## MATERIALS AND METHODS

### *Plant material*

*Crataeva nurvala*, locally known as Varuna was collected from forest areas of Keonjhar district of Odisha, identified and classified in the Department of Botany, Orissa University of Agriculture and Technology following the description of Saxena and Brahman<sup>21</sup>. The plant leaf after collection at pre-flowering stage was cleaned, dried under shade and ground into fine structure for preparation of extracts.

### *Solvents*

Solvents of extraction was classified into seven groups such as Gr-A (100% methanol), Gr-B (100% de-ionised water), Gr-C (methanol: water: acetic acid in 70:25:05 v/v), Gr-D (ethyl acetate: methanol: water in 60:30:10 v/v), Gr-E (100% acetone), Gr-F (acetone: water: acetic acid in 90: 9.5: 0.5 v/v) and Gr-G (100% ethanol)

### *Extraction*

Extraction of phyto-constituents was done in Microwave Assisted Extraction (MAE) system by Multiwave 3000-801V (Anton Paar) digestion system following the method of Senapati *et al.*, 2013<sup>22</sup> where 2 g of ground mass in 20ml of solvent was heated at 80<sup>0</sup> C for 25 minutes followed by 15 minutes cooling. During the extraction the initial temperature was 27<sup>0</sup> C-34<sup>0</sup> C with initial pressure between 2.6 to 3.7 bar. After cooling the temperature was between 30<sup>0</sup> C-40<sup>0</sup> C and pressure was 4.8 to 5.0 bar. The extracts were separated from the residues by filtering through Whatman No. 1 filter paper.

### *Removal of Chlorophyll*

Equal volumes of filtered crude extract and hexane were mixed and kept for 2 minutes. The supernatant was aspirated carefully to obtain chlorophyll free extract.

### *Estimation of total polyphenols*

Total polyphenol in the extract was determined by the method of Singh *et al.*, 2002<sup>23</sup>.

### Antioxidant activity Assay

The anti-oxidant activities were assayed with use of the concerned extract obtained from 1 g of leaf powder. The total antioxidant activity of extracts under different solvents was evaluated by phospho-molybdenom<sup>24</sup>, FRAP<sup>25</sup> and reducing power method<sup>26</sup>. Individual antioxidant capacities of these extracts were estimated by scavenging of Superoxide (SO)<sup>27</sup>, Nitric oxide (NO)<sup>28</sup>, and Hydroxyl radical (OH)<sup>29</sup>, whereas lipid per-oxidation inhibition assay (LPOIA) was estimated by the method of Ohkawa *et al.*, 1979<sup>30</sup>.

### Statistical analysis

The data was subjected to analysis of variance to test the significance of difference of mean values between different groups according to Snedecor and Cochran<sup>31</sup>.

## RESULTS:

### Total polyphenols content

The total polyphenol of chlorophyll free extract of *Crataeva nurvala* leaf in different solvents was estimated as exhibited in Table-1. Significantly higher ( $p < 0.01$ ) total polyphenols were observed in solvent mixtures of methanol with other solvents at Gr-D and C and in acetone at Gr-F as compared to other individual solvents at Gr-A, B, E and G. The values of total phenolics were in descending order from Gr-D > C > F > E > A > G > B.

### Total Antioxidant Activity

The polyphenols extracted in Gr-D exhibited significantly higher anti-oxidant activities ( $p < 0.01$ ) in phosphor-molybdenum and reducing power method than the solvents at Gr-C and F. The polyphenols at Gr-C solvent observed significantly higher activities ( $p < 0.01$ ) in FRAP method followed by Gr-D and F (Table-2).

### Individual Antioxidant/ Free radical scavenging Activity

The leaf polyphenols of *Crataeva nurvala* in all the test solvent groups exhibit radical scavenging of SO, NO and OH along with inhibition of LPO in a variable manner. The Gr-D solvent pose significantly higher SO and NO scavenging activities ( $p < 0.01$ ) followed by solvents of Gr-C and F whereas, OH scavenging activity and LPOI is significantly ( $p < 0.01$ ) higher in Gr-F solvents followed by that of Gr-C and D (Table-3).

**Table 1:** Total polyphenols of *Crataeva nurvala* leaf extracts in different solvent (Mean  $\pm$  SE) expressed as mg of Gallic acid equivalent per g of plant material (mg GAE / g of plant)

Gr-A	Gr-B	Gr-C	Gr-D	Gr-E	Gr-F	Gr-G
1.39 <sup>a</sup> $\pm$ 0.05	1.26 <sup>a</sup> $\pm$ 0.05	1.83 <sup>c</sup> $\pm$ 0.05	1.86 <sup>c</sup> $\pm$ 0.06	1.48 <sup>b</sup> $\pm$ 0.05	1.63 <sup>c</sup> $\pm$ 0.06	1.31 <sup>a</sup> $\pm$ 0.05

A: Methanol, B: Aqueous, C: Methanol + Water + Acetic acid, D: Ethyl acetate + Methanol + Water, E: Acetone, F: Acetone + Water + acetic acid and G: Ethanol, Means with different superscripts within rows showed significant difference ( $p < 0.01$ ) between the groups.

**Table 2:** Total Antioxidant activity of *Crataeva nurvala* leaf extracts (Mean  $\pm$  SE)

Methods	Gr-C	Gr-D	Gr-F
Phosphomolybdenum ( $\mu$ M of AAE / g of plant)	348.49 <sup>a</sup> $\pm 4.32$	487.58 <sup>b</sup> $\pm 4.33$	296.10 <sup>c</sup> $\pm 2.98$
FRAP ( $10^3 \times \mu$ M of AAE / g of plant)	7.03 <sup>a</sup> $\pm 0.11$	5.48 <sup>b</sup> $\pm 0.08$	2.40 <sup>c</sup> $\pm 0.06$
Reducing power ( $\mu$ M of AAE / g of plant)	744.05 <sup>a</sup> $\pm 3.21$	1061.29 <sup>b</sup> $\pm 3.33$	378.00 <sup>c</sup> $\pm 2.09$

C: Methanol + Water + Acetic acid, D: Ethyl acetate + Methanol + Water, F: Acetone + Water + acetic acid  
Means with different superscripts within the rows differ significantly ( $p < 0.01$ ).

**Table 3:** Individual Antioxidant (Radical Scavenging %) activity of *Crataeva nurvala* leaf extracts (Mean  $\pm$  SE)

Parameters	Gr-C	Gr-D	Gr-F
Superoxide (SO)	29.78 <sup>a</sup> $\pm$ 0.86	36.17 <sup>a</sup> $\pm$ 1.07	8.51 <sup>b</sup> $\pm$ 0.32
Nitric oxide (NO)	18.84 <sup>a</sup> $\pm$ 0.88	27.17 <sup>b</sup> $\pm$ 1.11	17.02 <sup>a</sup> $\pm$ 0.78
Hydroxyl (OH)	25.00 <sup>b</sup> $\pm$ 0.98	20.65 <sup>a</sup> $\pm$ 0.95	26.44 <sup>b</sup> $\pm$ 0.94
Lipid Per-oxidation Inhibition (LPOI)	15.94 <sup>a</sup> $\pm$ 0.82	15.21 <sup>a</sup> $\pm$ 0.95	20.28 <sup>b</sup> $\pm$ 0.85

C: Methanol + Water + Acetic acid, D: Ethyl acetate + Methanol + Water, F: Acetone + Water + acetic acid  
Means with different superscripts within the rows differ significantly ( $p < 0.01$ ).

## DISCUSSION:

Dietary polyphenols of fruits, vegetables, wine, tea, leaf, chocolate and other cocoa products are secondary metabolites / derivatives and include isomers of flavones, isoflavones, flavonols, catechins and phenolic acids. Over 8,000 structural variants of polyphenols with one or more phenolic hydroxyl groups attached to carbon-based aromatic phenyl-ring possess biological properties of anti-oxidants. Total phenolics contents vary between different plants, parts of flora within the same plant and solvents of extraction. The concentration of total phenolics are significantly lower ( $p < 0.01$ ) in water, methanol and ethanol extracts than Methanol + Water + Acetic acid mixture, Ethyl acetate + Methanol + Water mixture and Acetone + Water + acetic acid mixture. It depicts maximum components of polyphenols in mixtures of organic solvents than in aqueous and individual solvents. Synergistic effect of solvent polarity in mixture extracts significantly higher contents of polyphenols out of a wide variety. This may also be due to phyto-chemical diversity in extraction medium based on solubility of different constituents<sup>32</sup>.

Total anti-oxidant activity by phosphor-molybdenum assay is based on the reduction of  $\text{Mo}^{\text{VI}}$  to  $\text{Mo}^{\text{V}}$  by the antioxidant compounds and the formation of a green  $\text{Mo}^{\text{V}}$  complex with a maximal absorption at 695 nm. The reducing power of bioactive compounds is associated with antioxidant activity which is concomitant with the development of reducing power<sup>33</sup> and increases with increase in concentration of polyphenol<sup>32</sup>. Polyphenolics are good electron donors and terminate the radical chain reaction by converting free radicals to more stable products. The relevant chemical reaction of the FRAP method involves a single electron reaction between  $\text{Fe}(\text{TPTZ})_2$  (III) and a single electron donor  $\text{ArOH}$  where polyphenols like caffeic acid, tannic acid, ferulic acid, ascorbic acid, and quercetin, etc. react with  $\text{Fe}(\text{TPTZ})_2$  (III) to intervene the process. In the present study, the total anti-oxidant activities by Phosphomolybdenum and reducing power methods are higher in Ethyl acetate + Methanol + Water mixture (Gr-D) and lower in Acetone + Water + acetic acid mixture (Gr-F) which agrees to the findings of Halliwell and Gutteridge<sup>34</sup>. This may be due to more phenolics extracted in the solvent mixture with involvement of ethyl acetate with methanol by increasing their solubility. FRAP method estimates lower activity in comparison to other methods where Gr-C solvent exhibits higher values than Gr-D and E. It

corroborates with the findings of Pulido *et al.*, 2000<sup>35</sup>. This may be due to interference of extra-chlorophyll colour compounds of leaf other than Fe (TPTZ)<sub>2</sub> (II) in absorption at 593 nm under UV-Visible spectrum.

The scavenging of SO, NO, OH and inhibition of malondialdehyde (MDA) production are the avenues of chemical methods to evaluate individual anti-oxidative properties of plant phenolics. These radicals are generated on chemical induction of oxidative stress where sodium nitroprusside in standard phosphate buffer produces nitric oxide, Fenton systems produces hydroxyl radical and deoxy-ribose is degraded into MDA on exposure to hydroxyl radicals. MDA is produced on heating under acid conditions by reaction with thio-barbituric acid to form a pink chromogen<sup>36</sup>. Thio-barbituric acid reactive substances are produced as by-products of ferrous sulfate induced lipid per-oxidation and its inhibition is assayed for anti-oxidative activity. The leaf polyphenols of *Crataeva nurvala* in Gr-D solvent pose higher SO and NO scavenging activities than solvents of Gr-C and F but, OH scavenging activity and LPOI is more in Gr-F solvents than Gr-C and D. The observations of the present study is in agreement with the reports of reports of Gil *et al.*, 2000<sup>37</sup>, Veerapur *et al.*, 2009<sup>3</sup>, Karwani *et al.*, 2011<sup>14</sup> and Khatun *et al.*, 2012<sup>11</sup>. The radical scavenging and LPO inhibition of leaf polyphenols of *Crataeva nurvala* may be attributed to presence of diverged phenolic constituents extracted in different solvents. Since, the amount polyphenols and number of phenolics components vary between the solvent groups, variable radical scavenging and LPOI activities are observed by Gr-C, D and F solvents.

*Crataeva nurvala* leaf is a rich source of polyphenols which can be better extracted quantitatively in mixtures of Ethyl acetate + Methanol + Water and Methanol + Water + Acetic acid. The medicinal values of the plant leaf are based on its potent anti-oxidative effect against in vitro chemical induced free radical generation and lipid per-oxidation. Isolation, characterization and exploration of specific phenolic component responsible for such useful effect are recommended before further incorporation of the bio-active components in medicine.

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