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Assessment of anti-oxidant and wound healing potential of *Eclipta alba*, *Centella asiatica* and their combination with *Piper nigrum*

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Abstract

An attempt was made in the present studies, to arrive at some convincing correlation between the anti oxidant and wound healing potential of methanol extracts of *Eclipta alba* (ME), and *Centella asiatica* (MC), individually, and in presence of methanol extract of *Piper nigrum* (MP), as *Eclipta alba* and *Piper nigrum* (ME + MP), *Centella asiatica* and *Piper nigrum* (MC + MP), and in combination of *Eclipta alba*, *Centella asiatica* and *Piper nigrum* (ME + MC + MP) using albino rats. The significant results have justified the traditional use of these plants. The combinations of methanol extract of *Piper nigrum* significantly enhanced both anti-oxidant and wound healing potential of the test extracts suggesting its role as bio activity enhancer.

Key words: Methanol extract, *Eclipta alba*, *Centella asiatica*, *Piper nigrum*, anti-oxidant activity, wound healing.

1. Introductiona

The roots and leaves of *Eclipta alba* (*Asteraceae*) (*Bhringaraj*) and entire plant of *Centella asiatica* (*Umbelliferae*) (*Brahmi*) are effective in wound healing [1, 2]. *Eclipta alba* widely available Indian herb is an annual plant, with short, flat or round stem, deep brown in color, contains oleanane triterpene glycosides. Wedololactone and nor-wedelolactone [3] are major active constituents responsible for hepatoprotective [4], antihyperglycemic [5], immunomodulatory [6] and analgesic [7] activities. *Eclipta alba* is also used traditionally as popular liver tonic and in various chronic skin diseases. The leaf juice was found effective when applied externally to treat minor cuts, abrasions, and burns[8].

Centella asiatica syn. *Hydrocotyl asiatica* is an herbaceous perennial plant native to India, a slender trailing herb, rooting at the nodes with orbicular reniform leaves. The primary active constituents are triterpenoid compounds like α-amyrin derivatives, asiaticoside, and madecassside. An infusion of the plant is used in India to treat leprosy and other skin diseases. It is also widely used as diuretic, alternative and tonic [9, 10]. Three triterpene - asiatic acid, madecassic acid and asiaticoside of *Centella asiatica* posses wound healing activity [11,12,13].

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A number of ayurvedic formulations containing *Eclipta alba* and *Centella asiatica* in combination with *Piper nigrum* (Black pepper) are recommended in treatment of skin disorders. Further, it is also believed in ayurvedic practice that *Piper nigrum* helps in enhancing bioavailability. Piperine, an alkaloid of black pepper is well reported as an inhibitor of NADPH–dependent cytochrome P_{450} catalyzed reactions [14]. It is also reported that anti-oxidant activity of piperine is due to its ability to inhibit enzyme responsible for formation of free radicals [15].

Cutaneous wounding causes a depression in the overall anti oxidant status making it more vulnerable to oxygen radical attack. There are plenty of evidences which suggest that increased production of reactive oxygen species and ineffective scavenging play a crucial role in various skin lesions and in modulation of fibroblast proliferation. Thus, the present study was designed to assess the relationship between the anti oxidant potential and the wound healing potential of methanol extract of *Eclipta alba* and *Centella asiatica* alone, in combination, as well as in presence of methanol extract of *Piper nigrum* so as to evaluate the traditional claims. The study also provides justification for formulation of combination products.

2. Materials and methods

Plant material: Fresh herb of *Eclipta alba* and *Centella asiatica* were procured from the local market of Vadodara. The dried fruits of *Piper nigrum* were purchased from local medicine store. The plant materials were authenticated by comparing them with the specimens placed in the herbarium of Herbal Drug Technology Laboratory of Pharmacy Department, The M. S. University of Baroda (voucher specimen number EA/10/2004, CA/8/2004 and PN/12/ 2004). The procured plant materials were dried under shade; aerial parts were separated from the herb and powdered to obtain 60 mesh powder size.

Reagents: Methanol, ascorbic acid, riboflavin, sodium nitroprusside and ethylenediamine tetra-acetic acid (Qualigens, Mumbai), Curcumin, Nitroblue tetrazolium and Griess reagent (Hi media), DPPH (Sigma Aldrich, USA) were used.

Extracts: About 100 grams of each powdered drug was extracted with methanol in soxhlet apparatus at 70°C till complete extraction. The volume of the extract was reduced first using rotary evaporator, and then dried in oven at 50°C under vacuum. The extracts of *Eclipa alba* and *Centella asiatica* were then standardized as per reported methods [16,17].

2.1 In vitro anti-oxidant activity

Three reported methods, Nitric oxide free radical scavenging activity, Super oxide scavenging activity and DPPH free radical scavenging activity were followed to determine the antioxidant activity of selected combinations.

2.2 Sample preparation

Sample solutions each of 10 mg/ml of standardized methanol extract was prepared separately and mixed so as each combination will contain 20 % w/v MP.

2.3 Nitric oxide free radical scavenging activity [18]

Nitric oxide generated from sodium nitroprusside in aqueous solution at physiological pH interacts with oxygen to produce nitrite ions which were measured by the Griess reaction. Briefly, the reaction mixture (3 ml) containing sodium nitroprusside (10 mM) in phosphate buffered saline (PBS) and the extracts in different concentrations were incubated at 25°C for 150 minutes. Incubated samples were added with 0.5 ml of Griess reagent. The absorbance of the chromophore formed was measured at

546 nm. The percentage inhibition of nitric oxide generated was measured by comparing the absorbance values of control and test. Curcumin was used as reference compound.

2.4 Superoxide scavenging activity [19]

Superoxide scavenging activity of the extracts was determined by McCord and Fridovich's riboflavin photo reduction method [20], which depends on light induced super oxide generation by riboflavin and the corresponding reduction of nitroblue tetrazolium(NBT). The assay mixture contained different quantities of the extracts and ethylene diamine tetra acetic acid (12 mM), NBT (50 μM), riboflavin (2 μM) and phosphate buffer (50 mM, pH 7.8) to give a total volume of 3 ml. The tubes were uniformly illuminated with an incandescent light (40 Watts) for 15 minutes and thereafter the optical density was measured at 560 nm. The percentage inhibition by the extracts were evaluated comparing the absorbance values of control and experimental tubes. Ascorbic acid solution in the concentration range 1-10 μg/ml was used as a standard.

2.5 DPPH Free Radical Scavenging Activity [21]

DPPH-free radical scavenging activity was measured according to the reported procedure with slight modification. Briefly, different methanol extracts were mixed with equal volumes (75 μl) of 0.13 % methanolic solution of DPPH. The resulting solutions were thoroughly mixed and absorbance was measured at 516 nm. Decrease in absorbance in presence of test samples at different concentration was noted after 10, 20, and 30 minutes. The scavenging activity was determined by comparing the absorbance with that of control containing equal volumes of DPPH solution and methanol. Ascorbic acid in methanol in the concentration range of 10-35 μg/ml was used as standard.

2.6 In Vivo Wound Healing Activity (Excision Wound Model) [22]

Albino rats of Wistar strain of either sex weighing 180-220 gm were used for the experiment. Animals were randomly divided into seven different groups each of six animals. The back of the animal was shaved and washed with spirit. A circular area of 500 Sq. mm was marked out with Indian ink of the intra-scapular region and its full thickness excised with scalpel and scissor under ether anesthesia. The first group served as control, which was given the vehicle orally. Second, third, fourth and fifth groups were given methanol extract's combinations orally (the suspensions of desired concentration were prepared in water). Animals were treated with drugs daily, from zero to 20th post wounding day. The wound contractions were measured as total percentage reduction in wound area on alternate days. The progressive decrease in the wound area was monitored periodically by tracing the wound margin on a tracing paper and the area was assessed using graph paper.

2.7 Data Analysis

Results of EC_{50} values for *In vitro* anti oxidant activity are expressed as mean ± S.D. Differences between group means were assessed by one-way analysis of variance (ANOVA), followed by Tukey-Kramer Multiple Comparisons test and Dunnett multiple comparisons tests to assess the significance of the differences between individual groups. P values higher than 0.05 were considered insignificant.

3. Results

3.1 Anti-oxidant activity

Anti oxidant activity is expressed as decrease in absorbance of the samples at a different concentration levels. The results are tabulated in Table 1. EC_{50} was calculated, based on

concentration of the sample required that gives the 50 % decrease in the absorbance compared to that of control reading.

3.2 Nitric oxide anion radical

Significant nitric oxide scavenging activity was detected in all tested samples, the ME+MP combination had the greatest capacity to react with and quench nitric acid on weight basis followed by MC+MP, under the experimental conditions. The EC_{50} values of these combinations were comparable with that of curcumin. The extracts alone did not show any significant quenching property. In this model of study, EC_{50} values of ME + MP, MC + MP and ME + MC + MP were decreased by 49.18, 41.5 and 11.38 % as compare to ME, MC and ME + MC respectively.

3.3 Superoxide anion radical

The superoxide quenching activity of the samples was very less as compared to ascorbic acid. However, ME+MC+MP and ME+MC to some extent showed better scavenging activity. The EC_{50} values of ME+MP, MC+MP and ME+MC+MP decreased by 9.17, 7.28 and 33.96% as compared to ME, MC and ME+MC respectively.

Table 1. EC_{50} values of studied extracts found in *In vitro* anti-oxidant activity.

Group	Nitric Oxide scavenging activity (Curcumin as standard)	Super oxide scavenging activity (Ascorbic acid as standard)	DPPH Free radical scavenging activity (Ascorbic acid as standard)
	EC_{50} (µg) ± S.D.	EC_{50} (mg) ± S.D.	EC_{50} (µg) ± S.D.
Standard	2.20 ± 0.10	0.0131 ± 0.002	12.84 ± 0.23
ME	4.86 ± 0.1	6.17 ± 0.13	469.87 ± 6.28
ME + MP	2.47 ± 0.12	5.57 ± 0.16	361.93 ± 6.53
MC	4.41 ± 0.05	5.63 ± 0.36	448.37 ± 7.02
MC + MP	2.58 ± 0.16	5.22 ± 0.13	253.67 ± 5.69
ME + MC	3.69 ± 0.1	1.59 ± 0.17	273.53 ± 5.12
ME + MC + MP	3.27 ± 0.08	1.05 ± 0.06	235.20 ± 7.85

Values are mean ± standard deviation of three determinations.

Table 2. Effect of extracts of *Eclipta alba, Centella asiatica* and *Piper nigrum* on excision wound contraction in rat.

% closure of excision wound area after	Group						
days	Control	ME	ME + MP	MC	MC + MP	ME + MC	ME+MC+MP
4th	21.33±1.88	22.8±2.197	31.15±1.82$^{\#}$	36.48±1.52***	40.46±1.57**	26.24±1.10 $^{a, c}$	52.24±1.39^{+++}
8th	58.28±0.98	58.85±1.01	66.24±1.17$^{\#}$	66.46±0.78***	78.11±0.88**	68.11±0.85 $^{a, b}$	78.34±1.09^{+++}
12th	77.66±1.15	79.48±1.27	89.56±0.82$^{\#}$	90.46±1.8***	93.85±0.88**	82.2±0.973 $^{a, c}$	94.69±1.22^{+++}
16th	86.03±0.94	86.13±0.69	96.48±0.82$^{\#}$	98.29±0.92***	98.78±0.85**	92.29±1.47 $^{a, b}$	99.14±0.73^{+++}

Values are mean ± standard deviation of six readings.
$^{\#}$ P < 0.001 vs ME, ***P < 0.001 vs Control and ME, ** P < 0.01 vs MC, ^{a} P < 0.001 vs MC, ^{b} P < 0.001 vs ME, ^{c} P < 0.01 vs ME. $^{+++}$ P < 0.001 vs ME+ MC, ME+MP and MC+MP.

3.4 DPPH free radical

The kinetics were determined and it was found that ME+MC+MP had the greatest initial rate in reacting with DPPH and quenched the largest quantity of radicals in the system when the antioxidant-DPPH reaction reached equilibrium. MC+MP and ME+MC also showed higher scavenging activity. Results showed that EC_{50} values of ME + MP, MC + MP and ME + MC + MP were decreased by 22.97, 43.42 and 14 % as compare to ME, MC and ME + MC respectively.

3.5 Wound Healing Activity

There was no mortality observed in the course of study. The wound contraction and epithelialisation were faster in ME+MC+MP followed by MC+MP followed by ME +MP when compared to control. In the first two days after wounding, fluid was oozing from the untreated wounds (control) and to some extent, from ME+MC treated wounds. But in the other groups the drugs prevented the discharges from the wound. In the drug treated rats the wounds were completely healed in less than 18 days whereas in the control animals it took more than 26 days. Even on the 8th day the wound contraction was 78% in the treated rats (ME+MC+MP) whereas it was only 58 % in the control. The area of wound in control and treated groups are illustrated in Table 2. Statistical analysis showed very encouraging results. When compared with control and ME alone, MC showed highly significant activity ($P<0.001$). A significant activity of ME+MC was observed, when compared to ME ($P<0.01$) and MC ($P<0.001$) separately. In the similar manner ME+MP group showed highly significant activity in comparison to ME alone ($P<0.001$), while MC+MP showed significant activity when compared with MC alone ($P<0.01$) and ME+MC+MP showed highly significant activity when compared with ME+MC ($P<0.001$).

4. Discussion

Molecular oxygen plays a central role in the pathogenesis and therapy of chronic wounds. Overproduction of reactive oxygenspecies (ROS) results in oxidative stress thereby causing cytotoxicity and delayed wound healing. Therefore, elimination of ROS could be an important strategy in healing of chronic wounds [26]. Antioxidants hasten the process of wound healing by destroying the free radicals. It is therefore relevant to estimate antioxidant potential of plant extracts by *in vitro* studies and correlate it with their wound healing activity [27].

Alcohol crude extract of *Piper nigrum* is potential antioxidant, free radical scavenger [23], and bio-availability enhancer [24, 25]. The methods employed for determining anti oxidant activity indicated that the extracts when used in combination with methanol extract of *Piper nigrum* fruits, have shown better performance rather than used singularly. These studies thus provide support to consider methanol extract of *Piper nigrum* for synergistic effect on *in vitro* anti oxidant activity of ME and MC alone and in their combination.

The bioactivity of flavonoid is tightly correlated with their chemical structure and action mechanisms, mostly inhibitory on enzymatic systems involved in cellular activations [28]. Better collagenation seen under the influence of these plant extracts, may be because of the presence of phenolics and flavonoids [29-33], which is responsible for the free radical scavenging activity and is believed to be one of the most important components of wound healing [34]. The addition of MP in extract or combinations of extracts of ME and/or MC improves both in vitro anti oxidant activity and *in vivo* wound healing activity. The present studies reveal that the anti oxidant activity can directly be correlated with wound healing property of plant extracts.

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