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Immunostimulant Effects of Essential Oils of Peppermint and Eucalyptus in Chickens

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ABSTRACT

The immunostimulatory effects of peppermint and eucalyptus essential oils on cell mediated and/or humoral immunity in chickens vaccinated against Newcastle disease (ND) and Avian Influenza (AI) were investigated. The HI titers against both ND and AI vaccines were higher in volatile oils treated group as compared to the untreated control. The protection percentage reached 35% in volatile oils treated group as compared to 25% in untreated control group and 0% in unvaccinated negative control group on challenge with velogenic viscerotropic ND virus. The positive effect of volatile oils on phagocytic activity of macrophages was significantly ($P<0.05$) enhanced in treated birds over untreated ones at 14, 28 and 42 days of age. Their effect on serum nitric oxide level revealed significant increase in treated birds versus non-treated ones at 28 and 42 days of age. Lysozyme activity did not show significant change between treated and non-treated groups. In treated birds, scoring of histomorphological changes of major immune organs revealed lymphocytic hyperplasia and activation in Bursa of Fabricious, thymus, spleen and caecal tonsils with a sum of activation score reaching 8 versus 0 in untreated birds. It was concluded that eucalyptus and peppermint oils proved to be able to implement innate-cell mediated, humoral immune response and have a potent immunomodulatory effect in chickens.

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INTRODUCTION

It is well known that many diseases/disorders, that have immunomodulated components, can be modified by administration of biological compounds that activate key pathways in the immune system. They strengthen the defense and immune mechanisms of the body and can be used for stimulating the non-specific immune responsiveness in both the human and veterinary medical practice (Awaad *et al*., 1999, 2000a, 2000b).

Natural volatile oils are extensively used in cosmetics as well as in folk medicine for the treatment of many specific pathological conditions. Recently, the clinical use of essential oils has expanded worldwide, including treatment of various kinds of disease conditions such as allergy, asthma, rheumatism and arthritis (Leonard, 2004; Page, 2004). The antibacterial effects of eucalyptus leaf extract on pathogenic bacteria isolated from patients with respiratory problem have also been documented (Salari *et al*., 2006).

Similarly, Ocak *et al*. (2008) found a high growth promoting efficacy in peppermint leaves. Barbour (2006) evaluated the impact of eucalyptus and peppermint essential oils in the protection of the respiratory system of broilers against controlled challenges by *Mycoplasma gallisepticum* and/or avian influenza virus H9N2. However, no data are available about the influence of eucalyptus and peppermint essential oils on cellular components of the immune system. We investigated in this study whether these oils are able to stimulate either cell mediated and/or humoral immunity in chickens.

MATERIALS AND METHODS

Experimental design

A total of 150 one-day-old broiler chickens were randomly divided into 3 equal groups. All birds were kept in separate pens and fed on a commercial ration *ad libitum*. Chickens of all groups were vaccinated with

inactivated H5N2 Avian Influenza (AI) vaccine (Intervet International BV Boxmeer-Holland) by subcutaneous route at 7th and 28th day of age. Chickens of all groups were similarly vaccinated against infectious bursal disease at 14th day of age via eye drop route. For this purpose, a freeze-dried live vaccine Noblis Gumboro 228E (serial # 038916E, Intervet International, BV Boxmeer-Holland) grown on embryonated eggs having at least 2.0 $\log^{10}$ EID_{50} was used.

Birds of groups 1 and 2 were vaccinated against Newcastle disease (ND) with Hitchener B1+H120 vaccine (serial # 2137/01, Schering-Plough Animal Health, Millsoro, Delaware, USA) at 7th day of age and with LaSota ND vaccine (B1 type, LaSota strain) (serial # 1085208A, Fort Dodge Animal Health, Iowa 50501, USA) at 14th day of age by intraocular route. Chickens of group 1 only were orally treated with eucalyptus and peppermint essential oils in a dose of 0.25 ml/L of drinking water for 12 hours/day at 9-11, 16-18 and 23-25 days of age. Eucalyptus and peppermint essential oils in emulsifiers produced commercially under the trade name "MENTOFIN®" (Ewabo Co., Germany) were used. Chickens in group 3 were not vaccinated against NDV and served as negative control for ND.

Blood collection

Blood samples with and without anticoagulant were collected from wing veins of 5 randomly selected birds from each group at 14, 28 and 42 days of age. From fresh blood monocytes were separated for the measurement of phagocytic activity. Serum was separated from blood collected without anticoagulant and used for measurement of nitric oxide production and lysozyme activity.

Measurement of phagocytic activity

Measurement of phagocytic activity of peripheral blood monocytes using *Candida albicans* was adopted as described by Anthony *et al*. (1985) and Chu and Dietert (1989). Separation of peripheral blood mononuclear cells using ficoll-hypaque density gradient was carried out as described by Boyum (1968). Mononuclear cell layer was collected, washed and re-suspended in RPMI-1640 supplemented with 10% foetal calf serum and viability was recorded after Hanks and Waalace (1985). Phagocytic percentage and index were estimated as follows:

Phagocytic % =

No. of macrophages ingesting *Candida albicans*
---------- X 100
Total No. of macrophages

Phagocytic index =

No. of macrophages ingesting more than 3 blastopores

Total number of macrophages with ingested blastopores

Measurement of nitric oxide production

Nitric oxide production was determined according to Pertile *et al*. (1995). Briefly, 100 µl of serum sample was transferred into flat-bottom 96-well ELISA plates and 100 µl Greiss reagent were added to each well. The absorbance was read at 570 nm with ELISA plate reader, and then the absorbance was converted to µM of nitrite by comparison with absorbance values of sodium nitrite standard curve fit.

Lysozyme activity

The lysozyme activity was determined according to Schltz (1987) using agarose gel lysis assay. Briefly, lyso plates were prepared by dissolving 0.1% agarose in 0.06 M of PBS at pH 6.3, 500 mg of *Micrococcus lysodeikticus* in 5 ml saline were added to 1 litter of agarose. In lyso plates, 25 µl of serum samples and standard lysozyme were put in each well. After 18 hours, the diameter of cleared zones was measured for both standard lysozyme and serum sample and the concentration was estimated.

Evaluation of immunostimulating effect

To investigate the possible effect of eucalyptus and peppermint oils on humoral immunity, immunoassay was carried out. For this purpose, blood samples were collected from wing vein from 10 randomly selected birds at weekly intervals (1-6 weeks of age) from each group and serum samples were subjected to HI test for determining antibody titers against ND employing 8 HA, units as described by Swayne *et al*. (1998). Serum samples collected from birds of groups 1 and 2 were also subjected to HI test for determining antibody titers against Avian Influenza employing 4 HA units, as described earlier (OIE, 2004).

Bioassay against Newcastle disease virus

For bioassay against Newcastle disease virus (NDV), a velogenic viscerotropic strain of Newcastle disease (VVND) characterized previously was obtained from the Newcastle Vaccine Research Department, Veterinary Serum and Vaccine Research Institute, Abbasia, Cairo, Egypt and used in phases I and II. The challenge dose was 106.8 EID_{50}/ml/bird by intramuscular injection. The velogenicity of the virus was monitored by injecting 10 birds with a dose of $10^{6.8}$ EID_{50}/ml/bird intramuscularly which resulted in 100% mortality. At 28 days of age, 10 birds from each group were challenged with VVND and were kept under close observation for further 2 weeks for clinical signs, mortality and lesion scoring.

Histopathology

Five chickens from each group were sacrificed at 3, 7 and 10 days post IBDV vaccination. The bursa/body weight ratios were calculated. Bursae of Fabricious, spleen, thymus and caecal tonsils collected from the sacrificed 5 chickens per group were fixed in 15% buffered formalin, paraffin-embedded sections were stained with hematoxylin and eosin (Bancroft *et al*., 1996) and scored for histopathological lesions according to the method described by Rosales *et al*. (1989). Bursae were subjectively scored as 1 = no lesions, 2 = focal, mild cell necrosis or depletion, 3 = multifocal, 1/3 to ½ of the follicles showing atrophy and 4 = diffuse atrophy of all the follicles.

Statistical analysis

The obtained data were statistically analyzed using analysis of variance and comparison between groups was

performed using least significant difference (LSD) according to Petrie and Waston (1999) and computerized using SPSS (1999).

RESULTS AND DISCUSSION

Immunological studies

Tables 1 and 2 show the immune response against Avian Influenza and ND vaccination as well as results of vvNDV challenge of treated and untreated chickens with eucalyptus and peppermint oils. In general, the HI titers were higher in volatile oils treated group compared to untreated group. The protection percentage was 35% in volatile oils treated group as compared with 25% in untreated group and 0% in unvaccinated negative control group when challenged with vvNDV. Table 3 shows bursa/body weight ratios. The ratios were significantly ($P<0.05$) higher in volatile oils treated birds compared with untreated group at different examined intervals (21 and 24 days of age).

The positive effect of volatile oils on phagocytic activity has been illustrated in Table 4 that revealed significant increase ($P<0.05$) in serum nitric oxide level in treated group as compared to non-treated group at 28 and 42 days of age. Moreover, the phagocytic activity of macrophages was also enhanced ($P<0.05$) in treated birds over non treated ones at 14, 28 and 42 days of age. However, serum lysozyme activity did not show significant changes between treated or non-treated groups under the conditions of the present study.

Serum nitric oxide is one of the end products produced by macrophages as a result of their exposure to microbial products or chemotactic agents, the presence of nitric oxide in appropriate concentration during inflammation leads to immunomodulatory functions of host defense (Florquin *et al*., 1994). Phagocytes (macrophages) are known to play an important role in resistance to infection. They are part of the nonspecific first line of defense because of their ability to engulf and degrade invading microorganisms. Tizard (1996) and Stafford *et al*. (2002) reported that macrophages perform a variety of functions other than phagocytosis; they act as secretory cells, produce nitric oxide that kills intracellular microorganisms and also secrete many different proteins such as lysosomal enzymes and cytokines that play a key role in regulating immunity. Aderem and Underhill (1999) reported that internalization by macrophages occurs by a restricted number of phagocytic receptors present on their surface. Serafino *et al.* (2008) found that eucalyptus oil induces morphological and functional activation of human monocyte-derived macrophages. In our view, the stimulatory effect of eucalyptus oil observed in this study might be attributed to the stimulation of the complement receptor mediated phagocytosis. Nickels (1996) mentioned that peppermint oil maintains the structural integrity of immune cells due to its strong antioxidant action which protects cell membrane from free radical oxidants, thereby resulting in an improved immune response. According to Mekay and Blumberg (2006), peppermint oil has a significant antimicrobial, antitumor, antiviral, immunomodulating and chemopreventive potential. Iscan *et al*. (2002) and Schuhmacher *et al*. (2003) reported that peppermint oil had also antimicrobial effect against wide range of bacteria which improves the general healthy conditions of animal that may be reflected in increased immune response.

Table 1: HI antibody titers (HI geometric means) against Avian Influenza vaccine in birds treated with and without eucalyptus and peppermint oils

Age of birds (Days)	Groups	
	Eucalyptus and peppermint oils treated	Untreated control
7	7.3 ± 0.4^a	7.3 ± 0.4^a
14	3.0 ± 0.5^a	4.4 ± 0.3^b
21	4.0 ± 0.2^a	3.6 ± 0.4^a
28	5.2 ± 0.7^a	4.6 ± 0.6^a
35	4.6 ± 0.3^a	4.4 ± 0.2^a
42	6.6 ± 0.1^a	7.6 ± 0.5^b

Values in the same row with different superscripts are significantly different ($P \leq 0.05$).

Table 2: HI antibody titers (HI geometric means) against Newcastle disease virus (NDV) vaccine and protection against VVNDV in birds treated with and without eucalyptus and peppermint oils

Age of birds (Days)	Groups		
	NDV vaccinated and treated	NDV vaccinated and untreated	Unvaccinated negative control
7	$6.6 + 0.5^a$	$6.6 + 0.5^a$	$6.6 + 0.5^a$
14	$4.8 + 0.6^a$	$3.7 + 0.4^b$	$3.6 + 0.2^b$
21	$5.8 + 0.4^a$	$3.0 + 0.3^b$	$2.4 + 0.5^c$
28	$6.0 + 0.7^a$	$4.0 + 0.6^b$	$1.5 + 0.2^c$
Protection (%)	35.0	25.0	0.0

Values in the same row with different superscripts are significantly different ($P \leq 0.05$).

Table 3: Bursa/body weight ratios in birds treated with and without eucalyptus and peppermint oils

Age of birds (Days)	Groups	
	Eucalyptus and peppermint oils treated	Untreated control
17	$4.7 + 0.7^a$	$4.2 + 0.8^a$
21	$5.3 + 0.3^a$	$4.3 + 0.9^b$
24	$5.3 + 0.8^a$	$3.0 + 0.4^b$

Values in the same row with different superscripts are significantly different ($P \leq 0.05$).

Histopathological findings

Bursa of Fabricious

In eucalyptus and peppermint oils treated group (group 1), the 1st sample post vaccination (PV) revealed slight lymphocytic depletion in some lymphoid follicles. Second and 3rd samples PV showed lymphocytic hyperplasia and lymphoblasts activation (Fig. 1). In untreated group (group 2), bursae showed slight lymphocytic depletion, especially at the medulla of lymphoid follicles all over the experimental period (Fig. 2).

Table 4: Effect of eucalyptus and peppermint oils on cell mediated immunity in birds treated with and without eucalyptus and peppermint oils

Age of birds (Days)	Test	Groups	
		Eucalyptus and peppermint oils treated	Untreated control
14	Phagocytic %	50 ± 3.4^a	42 ± 1.3^b
	Phagocytric index	0.25 ± 0.08^a	0.19 ± 0.01^b
	Lysozyme activity (µg/L)	24 ± 8^a	17 ± 4^a
	Nitric oxide (µm/L)	77 ± 14^a	73 ± 8^a
28	Phagocytic %	52 ± 2.9^a	41 ± 1.0^b
	Phagocytric index	0.23 ± 0.08^a	0.18 ± 0.08^b
	Lysozyme activity (µg/L)	27 ± 5^a	26 ± 1.4^a
	Nitric oxide (µm/L)	95 ± 15^a	45 ± 6^b
42	Phagocytic %	50 ± 3.1^a	41 ± 1.4^b
	Phagocytric index	0.21 ± 0.02^a	0.16 ± 0.06^b
	Lysozyme activity (µg/L)	63 ± 9^a	63 ± 10^a
	Nitric oxide (µm/L)	66 ± 13^a	43 ± 14^b

Values in the same row with different superscripts are significantly different ($P \leq 0.05$).

Table 5: Scoring of histomorphological changes in major immune organs in birds treated with and without eucalyptus and peppermint oils

Histomorphological changes	Groups	
	Eucalyptus & peppermint oils treated	Untreated control
Activation scores		
B.F. Lymphocytic activation	1	0
B.F. lymphocytic hyperplasia	1	0
Thymus lymphocytic hyperplasia	1	0
Spleen lymphoblasts activation	1	0
Caecal tonsils lymphoblasts activation	2	0
Caecal tonsils lymphoytic hyperplasia	2	0
Sum of activation score	8	0
Histopathological lesions scores		
B.F. lymphocytic depletion	0	1
Thymus lymphocytic depletion	0	1
Thymus focal hemorrhage	0	2
Caecal tonsils lymphocytic depletion	0	1
Sum of histopathological lesion scores	0	5

Histopathologic assessments of the experimental parameters were graded as follows: 0 showing no changes; 1, 2, and 3 indicating mild, moderate and severe changes, respectively: B. F. = Bursa of Fabricious

Thymus glands

In group 1, slight lymphocytic hyperplasia (Fig. 3) was observed all over the experimental period. The untreated group (group 2) showed similar changes in the collected samples, these changes include slight lymphocytic depletion in the thymus medulla (Fig. 4) together with thymic haemorrhages (Fig. 5).

Spleen

In group 1, the 1st sample of spleen PV revealed hyperplasia of reticular cells and slight lymphocytic depletion (Fig. 6). Second and 3rd samples PV showed no histopathological changes except lymphoblasts activation (Fig. 7). In untreated group, examined sections revealed no histopathological changes throughout the experimental period.

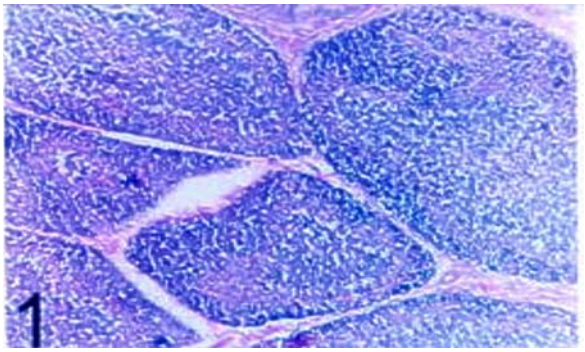

Fig. 1: Bursa of Fabricious of chicken from group 1 (3rd sample PV) showing lymphocytic hyperplasia and lymphoblasts activation (H & E, X 100).

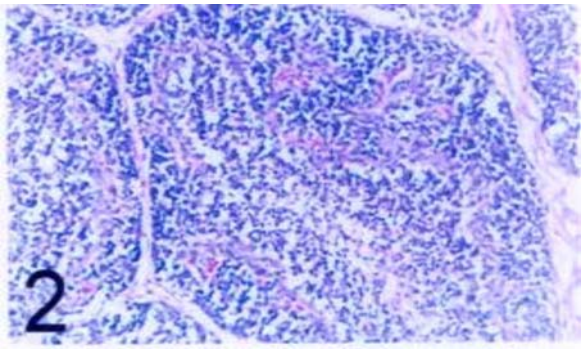

Fig. 2: Bursa of Fabricius of chicken from group 2 (2nd sample PV) showing lymphocytic depletion especially at the medulla of lymphoid follicles (H & E, X 200).

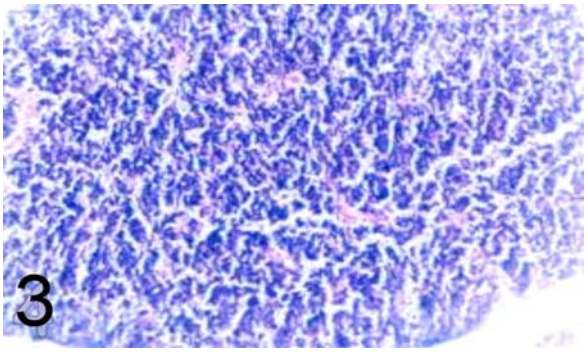

Fig. 3: Thymus gland of chicken from group 1 (2nd sample PV) showing slight lymphocytic hyperplasia (H & E, X 200).

Caecal tonsils

In group 1, caecal tonsils showed lymphoblastic activation and lymphocytic hyperplasia (Fig. 8), these lesions were noticed throughout the experimental period. Slight lymphocytic depletion (Fig. 9) was the only histopathological finding observed in sections from chickens in untreated group (group 2).

Scoring of the histomorphological changes of the major immune organs clarified lymphocytic hyperplasia and activation in Bursa of Fabricious, thymus glands,

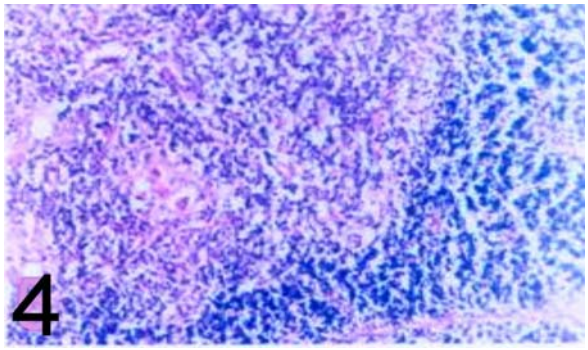

Fig. 4: Thymus gland of chicken from group 2 (2nd sample PV) showing slight lymphocytic depletion in the thymic medulla (H & E, X 200).

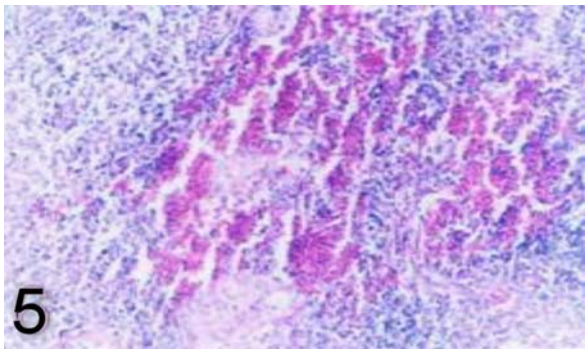

Fig. 5: Thymus gland of chicken from group 2 (2nd sample PV) showing focal thymic hemorrhages (H & E, X 200).

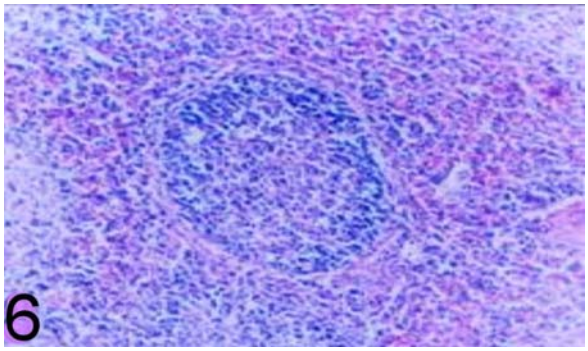

Fig. 6: Spleen of chicken from group 1 (2nd sample PV) showing lymphoblasts activation (H & E, X 200).

spleen and caecal tonsils with a sum of activation score reaching 8 versus 0 in untreated chicken group. Contrary to this, histologically there was depletion in these organs in the untreated group reaching a score of 5 as compared to 0 in the treated group (Table 5).

Thus, our data indicate that eucalyptus and peppermint oils are able to implement both innate-cell mediated and humoral immune response in chickens. It could be concluded that administration of these volatile oils has a potent immunomodulatory effect and evoke the immune response in chickens. This conclusion confirms results obtained by Barbour and Danker (2005), who reported that essential oils of eucalyptus and peppermint improved the homogeneity of immune responses and performance in MG/H9N2-infected broilers.

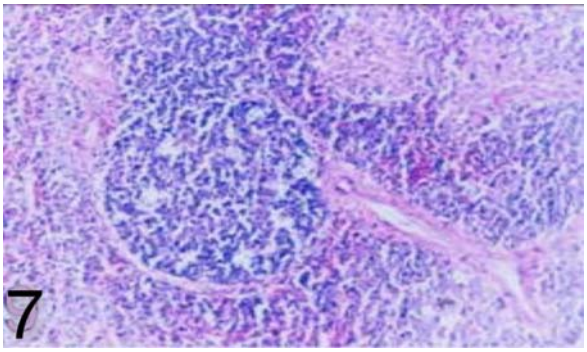

Fig. 7: Spleen of chicken from group 2 (2nd sample PV) showing no histopathological changes (H & E, X 200).

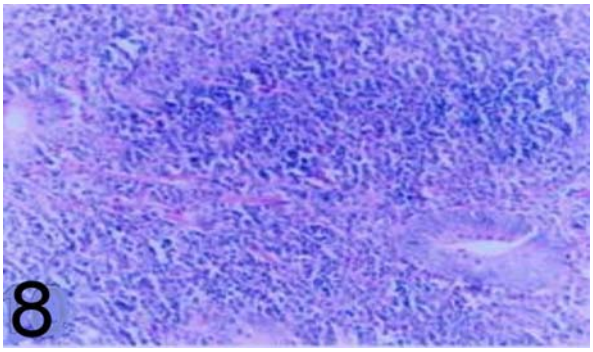

Fig. 8: Caecal tonsils of chicken from group 1 (2nd sample PV) showing lymphoblasts activation and lymphocytic hyperplasia (H & E, X 200).

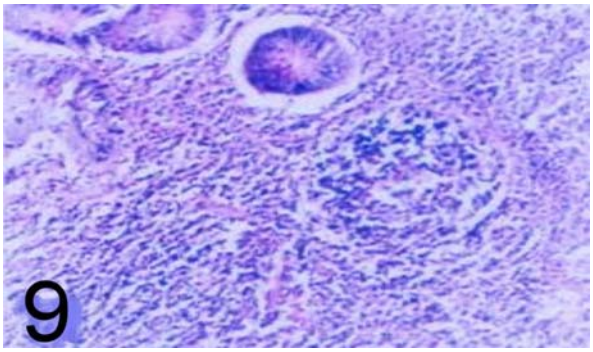

Fig. 9: Caecal tonsils of chicken from group 2 (3rd sample PV) showing slight lymphocytic depletion (H & E, X 200).

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