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# Clinical Evaluation of Phyto-formulation for Canine Distemper Virus by ELISA

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**Abstract**

Canine Distemper virus (CDV) is a highly contagious and devastating infectious disease in canines, with higher incidence in young and unvaccinated puppies. Controlling CDV outbreaks is a challenge in developing countries especially for free-roaming dogs (FRDs) due to low CDV vaccinations. This study aimed to investigate the efficacy of a phyto-formulation consisting of *Andrographis paniculata, Curcuma longa,* and *Tinospora cardifolia* extracts against CDV in clinically infected dogs. Dogs presenting suggestive symptoms of CDV such as increased body temperature, ocular and nasal discharge, seizures, temporal twitching, and digestive disturbances were included in the study. A randomized controlled clinical trial was conducted, with dogs categorised into two groups: a control group received standard antibiotics therapy and supportive treatment, and treatment group received phytoformulation. Clinical signs were monitored throughout the study period. The phyto-formulation demonstrated potential action in reducing the severity of the clinical signs associated with CDV infection. Antigen ELISA tests revealed a decrease in the serum antigen levels in the treated groups compared to the control group on day 7 ($0.45 \leq 0.01$ vs. $0.59 \leq 0.01$), day 14 ($0.37 \leq 0.001$ vs. $0.67 \leq 0.001$), day 21 ($0.28 \leq 0.001$ vs. $0.62 \leq 0.001$) respectively. The phyto-formulation comprising *Andrographis paniculata, Curcuma longa*, and *Tinosporacardifolia* extracts demonstrated ameliorative potential in mitigating the clinical signs and modulating the immune response against CDV in infected dogs.

Worldwide, canine population are infected by highly consequential and devastating infectious disease known as the canine distemper (CD) (Headley *et al.* 2000, Martella *et al.* 2008 and Perez *et al.* 2010). The global prevalence of CDis increasing and presents ongoing challenges in underdeveloped countries, particularly in terms of managing outbreaks and controlling the population of free-roaming dogs (FRDs) (Rikula *et al.* 2007, Jackman *et al.* 2007, Acosta-Jamett *et al.* 2011 and Dalla *et al.* 2010). Regrettably, the CD vaccination rates in these nations are typically insufficient to create strong enough herd immunity (Jackman *et al.* 2007). In urban areas, high densities of FRDs can perpetuate CD virus (CDV) within their population, presenting significant risks to themselves and other susceptible wild species. Additionally, environmental factors such as monsoon, high humidity, etc. will play an incumbent role in spreading out an endemic (Acosta-Jamett *et al.* 2010, Latha *et al.* 2007 and Blixenkrone-Moller *et al.* 1997).

Canine distemper is an acute, highly contagious multi-systemic infection caused by a virus belonging to the genus Morbillivirus in the Paramyxoviridae family. Dogs of all breeds and ages are susceptible, but younger ones aged <1 year and unvaccinated dogs are the most vulnerable group, as they lack passive immunity from maternal colostral antibodies. In addition to neurological symptoms like tremors, circling, head tilt, and convulsions, clinical signs of CD

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include loss of appetite, biphasic fever, enlarged mandibular lymph nodes, depression, ocular and nasal discharge, diarrhoea, vomiting, dyspnea, coughing, and pneumonia (Martella *et al.* 2008 and Ezeibe, 2005). Vaccination is the primary source of defense against CDV, and when clinical signs manifest, supportive care is the only treatment option.

In light of these circumstances, the plan was to evaluate the efficacy of the phyto-formulation which combines standardised extracts of *Andrographis paniculata*, and *Tinospora cardifolia* and rhizomes of *Curcuma longa*. These ingredients possess remarkable medicinal properties and were selected to enhance the immunity of canines against various infections, particularly CDV. *Andrographis paniculata*, belongs to the Acanthaceae family and is an annual herbaceous plant. It is widely utilized in Ayurveda, Siddha, and Unani systems of medicine for its hepatoprotective and immune-modulating properties, as well as its efficacy in treating infections. It has not only found to significantly alleviate symptoms associated with the common cold, such as fatigue, insomnia, sore throat, and nasal congestion but also possess good antibacterial and antiviral properties by neutralizing Human Immunodeficiency Virus (HIV) and Herpes Simplex Virus (HSV), inhibiting the expression of Epstein Barr-virus lytic protein, stimulating antibody protection and the innate immune response, inhibiting Nuclear Factor Kappa B activation, TNF-α and IL-12, and regulating the production of IFN γ and IL-2 (Subramanian *et al.* 2006 and Subramanian *et al.* 2008) .

Intensive studies are still being conducted on the curcuminoids (which is a medically useful ingredient) and yellow pigments in turmeric (*Curcuma longa* rhizomes). Numerous clinical, preclinical, and cell culture research throughout the past century have emphasised the significant biological roles like anti-cancer, radioprotective, neuroprotective, anti-inflammatory, and antioxidant effects (Amalraj *et al.* 2016). Studies have shown that curcuminoids can reduce the neuronal dysfunction and damage brought on by the HIV-gp120 V3 loop peptide, boost the expression of Brain Derived Neurotrophic Factor (BDNF), scavenge superoxide anions, protect against lipid peroxidation, prevent homocysteine neurotoxicity, and slow the progression of kindling in mice (Amalraj *et al.* 2016). Turmeric is commonly employed as a dietary supplement for animals such as chickens, dairy cows, goats, fish, and dogs due to its notable antibacterial, antifungal, and antiviral properties (Gupta *et al.* 2015).

Tinocordin, an active compound present in *Tinospora cordifolia*, has been demonstrated to increase leukocyte count and alleviate neutropenia. It enhances the phagocytic and intracellular killing capacity of macrophages and polymorphonuclear leukocytes, stimulates the release of cytokines such as granulocyte macrophage colony-stimulating factor, IL1, IL2, and promotes B lymphocyte proliferation (Sharma *et al.* 2010).

Our study aims to provide valuable insights on the use of natural compounds to enhance the immune response and mitigate the effects of CDV infection in dogs. The findings of this research may lead to the development of novel therapeutic approaches for CDV and other infectious diseases in canines, ultimately improving the overall health and well-being of these animals, thus making a positive impact on their lives.

## Materials and Methods

### Animals

Recruitment of canines was conducted at Veterinary dispensary in and around Chennai and Thiruvallur district. Client-owned dogs with CDV infection (from May 2023 to September 2023) were inducted into the randomised control trial study. Eligibility of the patients was assessed by the attending clinician, and canines exhibiting suggestive symptoms of CD were included. The clinician had discretion in determining the eligibility, and owners were informed about the study.

### Experimental Design

The phytoformulation (ACT) used in the study consisted of standardised extracts from *Andrographis paniculata*, *Curcuma longa* rhizomes, and *Tinospora cardifolia* (Devi *et al.* 2022). Based on Devi *et al.* (2022), phytoformulation as supportive treatment was used in CD affected dogs. A total of 22 dogs affected by CDV were randomly assigned to two groups: study groups (12 dogs) and a control group (10 dogs). The control group

received normal symptomatic and supportive treatment, including antibiotic therapy and fluid therapy for dehydration. Dogs in the treatment group received the antibiotic therapy mentioned earlier along with a daily oral administration of the Phytoformulation (ACT). For treatment group, the dosage was therapeutic dose of phytoformulation previously used (Devi *et al.* 2022) i.e. *Andrographis paniculata* leaf extract 0.6g/kg, *Curcuma longa* rhizomes extract 0.3g/kg, and *Tinosporacardifolia* leaf extract 0.1g/kg. The treatment was administered for 21 days, followed by a 7-day observation period. Clinical signs and blood samples were monitored from the day of presentation.

### CD confirmation by RT-PCR

RNA was isolated from blood using TRIZol reagent, and reverse transcription PCR (RT-PCR) was performed using previously published primers for the nucleoprotein gene of CDV (Elia *et al.* 2006). The PCR product was analyzed using agarose gel electrophoresis.

### Antigen ELISA

Antigen levels of PCR confirmed CD dogs during the course of treatment were assessed using Antigen ELISA. Serum samples diluted 1:5 in carbonate buffer were added to a 96-well plate coated with CDV-specific antibody (santacruz biotechnologies, USA). After incubation and washing steps, an HRP-conjugated anti CDV antibody (santacruz biotechnologies, USA) was added. Color development was observed using a substrate buffer, and absorbance was measured at 450 nm (Soma *et al.* 2003).

### Statistical Analysis

Statistical analysis was conducted using Prism GraphPad software 8.0. Data were expressed as mean ± SD, and statistical differences were assessed using one-way ANOVA. A p-value of less than 0.05 was considered significant (* $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$).Survival analysis of canine distemper animals with phyto treatment andwithout phyto treatment was done by construction of Kaplan Meier curveusing Prism GraphPad software 8.0.

### Results

In the study a total of 22 dogs were taken, including 10 controls. From the day of presentation, clinical signs were observed and graded in both a control and treatment animal group for up to 14 days with temperature, ocular or nasal discharge, seizure, temporal twitching, neuromuscular coordination, digestive disturbances.

### CD confirmation by RT-PCR

For the confirmation of CDV, a RT-PCR assay was performed. Fig 1 showed the formation of a nucleoprotein gene from CDV by RT-PCR.

### Clinical manifestations

A comparison of the results between the treatment group and the control group is presented in Table II. In the treatment group, a significant improvement was observed in the clinical symptoms. The average temperature decreased from 103±2°F on the 0th day to 102±2°F on the 3rd day and 101±2°F on the 7th day. The presence of oculo nasal discharge decreased from "+++++"(severe) to "+" (mild) over the same period. The number of animals experiencing seizures, temporal twitching, ataxia, and neuromuscular incoordination also decreased progressively. Furthermore, no digestive disturbances were observed on the 3rd and 7th days in the treatment group. In contrast, the control group exhibited consistently high temperatures and a continuous presence of oculo-nasal discharge. While the control group had minimal seizures, temporal twitching, ataxia, and neuromuscular incoordination were observed. Digestive disturbances increased in the control group over the

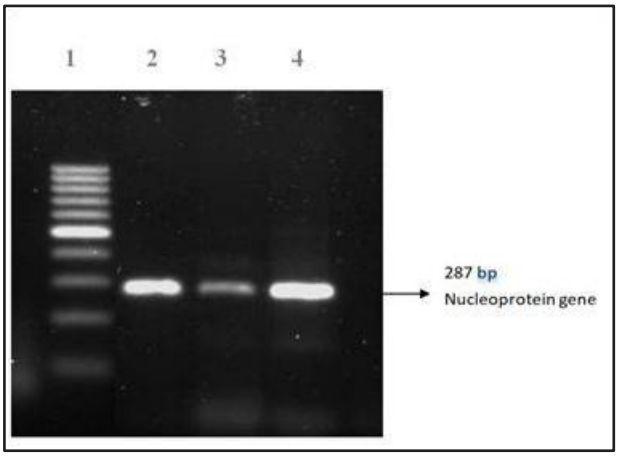


**Fig 1**: Amplification of nucleoprotein gene of CDV by RT-PCR
Lane1:100bp DNA molecular weight marker Lane2-4 : Nucleoprotein gene
amplicons

**Table I:** Grading of clinical signs in treatment group

| S.No. | Cardinal signs | 0 day | 3rdday | 7thday |
|---|---|---|---|---|
| 1 | Temperature | 103±2$^0$F | 102±2$^0$F | 101±2$^0$F |
| 2 | Presenceof ocular and nasal Discharge | +++++ | +++ | + |
| 3 | Presence of seizure (No. Of animals)-mild | 6 | 2 | 0 |
| | Moderate, severe | 4 | 1 | 0 |
| | | 2 | 0 | 0 |
| 4 | Presence of temporal twitching(No.Ofanimals)- | 5 | 3 | 3-reducedfrequency |
| 5 | Ataxia, neuromuscular incoordination (No. Ofanimals)- | 12 | 10 | 7-progressiveimprovementand controlledactivity/movements |
| 6 | Digestive disturbance, Vomition and diarrhoea (No.Of animals)- | 7 | - | - |

++++severe, +++moderate, ++mild

observation period, indicating a contrast with the improvements observed in the treatment group (Table II).

### Antigen ELISA

In the reduction phase, a decreasing trend in antigen concentration was observed in 10 out of 12 dogs from the study group compared to the control animals. The treatment group received ACT phytochemicals, including the extract of *Andrographis paniculata*, which exhibited antiviral activity and significantly inhibited viral antigen. From the Fig 2, antigen ELISA test revealed a decrease in CDV antigen levels in the treated groups compared to the control group on day 7 ($0.45 \leq 0.01$ vs. $0.59 \leq 0.01$), day 14 ($0.37 \leq 0.001$ vs. $0.67 \leq 0.001$), day 21 ($0.28 \leq 0.001$ vs. $0.62 \leq 0.001$) respectively.

### Survival analysis by Kaplan Meier curve

Survival analysis by Kaplan Meier curve implied that probablility of survival by phyto treatment curved well above the control animals without phyto treatment. These findings suggest that the treatment with phytoformulaton (ACT), containing *Andrographis paniculata*, *Curcuma longa*, and *Tinospora cardifolia* extracts resulted in a reduction in clinical symptoms, and viral antigen concentration.

### Discussion

Canine distemper is one of the dangerous infectious diseases with a high mortality rate in canines, and its clinical manifestation depends upon the host immune system (Kapil and Neel, 2015). So far, no approved antiviral drugs are available in the market against CDV infection. Vaccination is the only source available to protect the dogs from CDV to a certain extent. To tackle this problem, researchers are investigating many antiviral drugs (Carvalho *et al.* 2013), including several flavonoids, phenolic acids (Lanave*et al.* 2017), ribavirin (Wu *et al.* 2017), boceprevir (Devi *et al.* 2022), caffeic acid (Devi *et al.* 2022), homeopathic drugs (Krumm *et al.* 2014), and viral polymerase-targeting inhibitors (Krumm *et al.* 2014) to overcome the clinical symptoms of CD and to enhance the immune

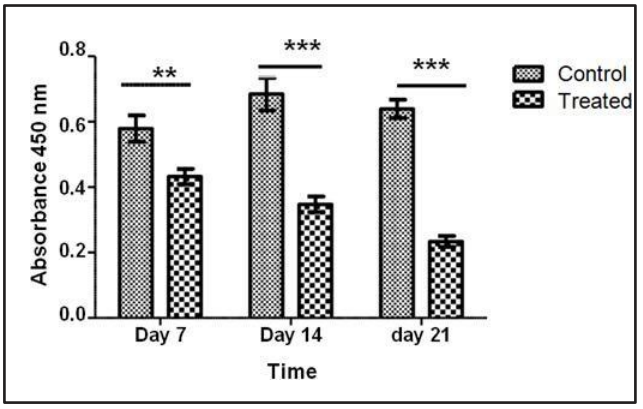


**Fig 2 :** Antigen ELISA was carried out to determine the titre of antigen in the serum of Dogs in treatment and control group on day7,14 and 21.(*P≤0.05**P≤0.01,***P≤0.001).

**Table II** : Grading of clinical signs in control group.

| S.No. | Cardinal signs | 0 day | 3rd day | 7th day |
|---|---|---|---|---|
| 1 | Temperature | 104.6$^0$F | 104.2$^0$F | 104.3$^0$F |
| 2 | Presence of ocular and nasal Discharge | +++++ | ++++ | ++++ |
| | Presence of seizure | | | |
| 3 | (No.Ofanimals)- Mild | 0 | 0 | 0 |
| | Moderate | 2 | 2 | 1 |
| | Severe | 1 | 0 | 1 |
| 4 | Presence of temporal twitching(No.Ofanimals) | 1 | 2 | 2 |
| 5 | Ataxia,neuromuscular incoordination (No. Ofanimals) | 1 | 2 | 2 |
| 6 | Digestive disturbance Vomition and diarrhoea | 1 | 2 | 2 |

++++severe,+++moderate,++mild

system of infected dogs or other animals.

Initially the disease was confirmed by RT- PCR (Sehata *et al.* 2015). The oral intake of phyto formulation resulted in significant improvements in the cardinal signs of the disease (Bagla *et al.* 2012). These improvements included a reduction in temperature, occulo-nasal discharge, and digestive disturbances. Furthermore, the severity of nervous signs, such as seizures, temporal twitching, ataxia, and neuromuscular coordination, was reduced compared to the control animals over a period of 14 days.

In this study, the early antigen titres of dogs affected by CDV were measured (Soma *et al.* 2003). Comparing them to the control animals during the test period, the majority of the study group dogs showed a decreasing trend in antigen titre, which can be attributed to the antiviral, anti-inflammatory and Immune enhancing properties of the phytoformulation ACT, that combines standardised extracts of *Andrographis paniculata, Tinospora cardifolia* and *Curcuma longa rhizome*. The levels of antigen and antibody titres in the serum of CDV-affected dogs were significantly influenced by the severity of the disease, the immune status of the animal, and vaccination history.

Most of the dogs in the study group were either not vaccinated or had received only a single vaccination as puppies. The antigen titre in vaccinated dogs was considerably lower when compared to the diseased dogs (Soma *et al.* 2003). The decreasing trend in antigen titre over the study period clearly indicates a reduction in viral load as the disease progresses.

In conclusion, the phytoformulation (ACT) containing standardised extracts of *Andrographis paniculata*, *Curcuma longa* rhizomes, and *Tinospora cordifolia*can be considered as a potential adjunctive therapy for canine Distemper virus (CDV)-infected dogs. The treatment group receiving the phytoformulation exhibited improved clinical symptoms, and inhibition of viral antigen. These findings suggest the phytoformulation's antiviral activity and immune response enhancement potential. Further research is needed to optimize dosages for optimal therapeutic outcomes. Implementing such alternative treatments, alongside comprehensive vaccination strategies, may help control CDV outbreaks and mitigate risks posed by free-roaming dogs, particularly in resource-limited regions. The phytoformulation holds potential for enhancing the health and well-being of dogs in both urban and rural settings.

## References

Acosta-Jamett,G,Chalmers,W.S.K.,Cunningham,A.A.,Cleaveland, S., Handel, I. G., and Bronsvoort, B. M., (2011). Urban domestic dog populations as a source of canine distemper virus for wild carnivores in the Coquimbo region of Chile. *Vet. Microbiol.* **152**: 247–257.

Amalraj, A, Pius A, Gopi, S, and Gopi, S. (2016). Biological activities of curcuminoids, other biomolecules from tur-

meric and their derivatives - A review. *J.Tradit.Complement.Med*.;**7**(2) : 205–233.

Blixenkrone-Moller, M, Svansson, V, Have, P, Orvell, C, Appel, M, Pederson, I. R, Dietz, H. H., and Henriksen, P. (1993). Studies on manifestations of canine distemper virus infection in an urban dog population. *Vet. Microbiol.* **37**: 163–173.

Carvalho, O. V., Botelho, C. V., Ferreira, C. G., Ferreira, H. C., Santos, M. R., Diaz, M. A., Oliveira, T. T., Soaresmartins, J. A., Almeida, Mr. Jr, and Silva, A. Jr. (2013). *In vitro* inhibition of canine distemper virus by flavonoids and phenolic acids: implications of structural differences for antiviral design. *Res Vet Sci.* **95**(2): 717–724.

Dalla Villa, P,Kahn, S, Stuardo, L, Iannetti, L, DiNardo, A, and Serpell, J. A., (2010). Free-roaming dog control among OIE-member countries. *Prev.Vet.Med.***97**: 58–63.

Devi, T, M, Asokkumar, M, Vijaya Bharathi, A, Ramesh, K. G. Tirumurugaan, R. Venkataramanan, and N, Pazhanivel. (2022). Prognostic Factorial Index for Dogs with Canine Distemper. *Ind. j of animal res.* B-5017 : 1-5. DOi:10.18805/IJAR.B-5017.

Elia, G,Decaro, N,Mrtella, V,Cirone, F,Lucente, M. S., Lorusso, E, DiTrani, L and Buonavoglia, C. (2006). Detection of canine distemper virus in dogs by real-time RT-PCR. *J.Virol.Meth.* **136**: 171–176.

Ezeibe, M. C. O., (2005). Canine distemper in local dogs in Nsukka, Nigeria.Vet.Rec.;**156**: 840–842.

Gupta, A, Mahajan, S, and Sharma, R. (2015). Evaluation of antimicrobial activity of Curcuma longa rhizome extract against *Staphylococcus aureus*. *Biotech.Rep*; **6**.

Headley S. A, and Graca, D. L., (2000). Canine distemper: Epidemiological findings of 250 cases. *Braz.J. Vet. Res.&Anim.Sci.* 37.

Jackman, J, and Rowan, A. (2007). Free-roaming dogs in developing countries: The benefits of capture, neuter, and return programs. In D..J. Salem & A.N. Rowan(Eds.),The state of the animals Washington, DC: Humane Society Press. 55-78.

Kapil, S, and Neel, T. (2015). Canine distemper virus antigen detection in external epithelia of recently vaccinated, sick dogs by fluorescence microscopy is a valuable prognostic indicator. *J Clin Microbiol.* **53**: 687-691.

Krumm, S. A., Yan, D., Hovingh, E. S., Evers, T. J., Enkirch, T., Reddy, G. P., Sun, A, Saindane, M. T, Arrendale, R. F, Painter, G. (2014) Orally available small-molecule polymerase inhibitor cures a lethal morbillivirus infection. *Sci Transl Med.* **6**(232 – 252).

Lanave G, Cavalli A, Martella V, Fontana T, Losappio R, Tempesta M, Decaro N, Buonavoglia D, Camero and M. Ribavirin, (2017). Boceprevir are able to reduce canine distemper virus growth in vitro. *J Virol Methods.* ; **248** : JVI.00521-00517.

Latha, D, Srinivasan, S. R., Thirunavukkarasu, P. S., Gunaselan, L, Ramadass, P, and Narayanan, R. B. (2007). Assessment of canine distemper virus infection in vaccinated and unvaccinated dogs. *Ind.J. Biotech.* **6**:35–40.

Martella, V, Elia, G, and Buonavoglia, C. (2008). Canine distemper virus. Veterinary Clinics of North America: *Small Anim. Pract.* **38**: 787–797.

Merete Blixenkrone-Moller, I. Rode Pedersen, B. Max, J. Appel, and Christian Griot. (1991). Detection of IgM antibodies against canine distemper virus in dog and mink sera employing enzyme-linked immunosorbent assay (ELISA*). J Vet Diagn Invest*.; **3**:3-9.

Perez, A. M., Marro, V, Schiaffino, L, Pirles, M, Bin, L, and Ward, M. P. (2008). Risk factors associated with canine distemper in Casilda, Argentina. In Proceedings of the 10th International Symposium on Veterinary Epidemiology and Economics, ViñadelMar, Chile 17–21.

Rikula, U, Nuotio, L, and Sihvonen, L. (2007). Vaccine coverage, herd immunity and occurrence of canine distemper from 1990–1996 in Finland. **25**: 7994–7998.

Sehata, G, Hiroaki, S, Toshihiro, I, Yoshitaka, I, Taichi, N and Eiji, O. (2015). Use of quantitative real-time RT-PCR to investigate the correlation between viremia and viral shedding of canine distemper virus and infection outcomes in experimentally infected dogs. *J. Vet. Med. Sci.* **77**(7):851–855.

Sharma, V, and Pandey, D. (2010). Protective Role of Tinospora cordifolia against Lead-induced Hepatotoxicity. *ToxicolIntl*, **17**(1):12–17.

Soma, T. H., Ishii, M, Hara, K, Ohe, L, Hagimori, Y, Ishikawa, A, and Taneno. (2003). Detection of canine distemper virus antigen in canine serum and its application to diagnosis, *Vet. Rec*. **153:**499-501.

Subramanian, R, Asmawi, M. Z., and Sadikun, A. (2008). In vitro $\alpha$-glucosidase and $\alpha$-amylase enzyme inhibitory effects of *Andrographis paniculata* extract and andrographolide. *Acta Biochimica Polonica.* **55(2):**391–398.

Subramanian, R, and Asmawi, M. Z., (2006). Inhibition of $\alpha$-glucosidase by *Andrographis paniculata* ethanol extract in rats. *Pharmaceutical Biology.* **44**(8):600–606.

Wu Zong-Mei, Yu Zhen-Jiang, Cui Zhen-Qiang, Peng Lu-Yuan, Li Hao-Ran, Zhang Chun-Lei, Shen Hai-Qing, Yi Peng-Fei, and Fu Ben-Dong. (2017). *In vitro* antiviral efficacy of caffeic acid against canine distemper virus. *Microbial Pathogenesis.* **110**:240–244.